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THE UNIVERSITY OF ALBERTA

A STUDY OF THE ANTIGENICITY AND IMMUNOGENICITY OF THE MEASLES VIRUS SURFACE PROTEINS

by

© MIKA J. MÄKELÄ

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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DEPARTMENT OF MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

EDMONTON, ALBERTA

FALL, 1987

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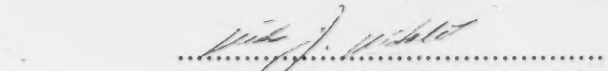

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled A STUDY OF THE ANTIGENICITY AND IMMUNOGENICITY OF THE MEASLES VIRUS SURFACE PROTEINS submitted by MIKA J. MÄKELÄ in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE IN VIROLOGY.

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ABSTRACT

In the present study, the antigenicity and immunogenicity of the surface proteins of the measles virus (MV) were analyzed. In the first part of the study, an assay for measuring polypeptide- and antigenic site-specific antibodies to fusion (F) and hemagglutinin (H) proteins of MV was developed. This method was used in analysis of sera from patients with different disease conditions. The results suggested that humoral responses to different components of MV are independent phenomena. Differences in relative antibody amounts between normal measles sera and sera from patients with chronic measles infection (subacute sclerosing panencephlalitis) were found indicating that persistent infection could elicit qualitatively different responses than regular infection.

Synthetic peptides were employed in characterization of the antigenicity of the MV H protein at a molecular level. Four peptides, designated H-5, H-6, H-9 and H-10, corresponding to regions predicted to be exposed on the surface of the H of Edmonston strain of MV were synthesized. Three of the peptides bound antibodies present in polyclonal serum raised against both purified virus and purified H indicating that these sites are exposed on the surface. Antibodies to the peptides were raised in rabbits and the antisera characterized with several immunological assays. All peptides elicited antibodies both to the homologous peptide and to the virus H protein although the immunogenicity of the H-5 peptide was clearly lower. The H-9 peptide elicited high amounts of protein-reactive antibodies and the recognition pattern obtained from different tests suggested that this determinant is exposed on isolated H.

Immunogenicity of the H component in T cell assays was studied in a mouse model. The results showed that the H was a major antigen in the proliferative assay and its immunogenicity depended on the form in which it was introduced to the cellular immune system. Two non-conjugated synthetic peptides were also used in this system and both were shown to prime mouse lymphocytes to a secondary challenge with MV. As the

region of the H component corresponding to one of these peptides, H-9, also has high immunogenicity in the humoral system, it was suggested that this determinant could be biologically significant in both arms of the immune system.

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ABBREVIATIONS

AG	antigen
APC	antigen presenting cell
BSA	bovine serum albumin
CAH	chronic active hepatitis
CDV	canine distemper virus
CF	complement fixation
Ci	curie
CNS	central nervous system
Con A	concanavalin A
CP	cyclophosphamide
CPE	cytopathic effect
CSF	cerebrospinal fluid
CTL	cytotoxic lymphocyte
DDA	dimethyl dioctadecyl ammonium bromide
DEAE	diethylaminoethyl
DOC	deoxycholate
DTH	delayed-type hypersensitive assay
EIA	enzyme immunoassay
F	fusion protein
FCA	Freund's complete adjuvant
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gravitational force
H	hemagglutinin
h	hour
HA	hemagglutination assay
HAU	hemagglutinating units
HI	hemagglutination inhibition
HLA	human leukocyte antigen
HLI	hemolysis inhibition
HN	hemagglutinin-neuraminidase
HR	high-responder
IF	immunofluorescence
IU	international unit
Kd	kilodalton
KLH	keyhole limpet hemocyanin
L protein	large protein
LL	lentil lectin
LR	low-responder
LS	liposome
M	matrix protein
M	molar
m.o.i.	multiplicity of infection
MAb	monoclonal antibody
µg	microgram
MEM	minimum essential medium
mg	milligram
µl	microliter
ml	milliliter
MS	multiple sclerosis
MV	measles virus

nm	nanometer
NBD	normal blood donor
NC	nucleocapsid
NP	nucleoprotein
NT	neutralization test
OD	optical density
P	polymerase protein
Pa	pascal
PBS	phosphate buffered saline
RIA	radioimmunoassay
RIPA	radio-immunoprecipitation assay
RSV	respiratory syncytial virus
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SSPE	subacute sclerosing panencephalitis
SV5	simian virus 5
v/v	volume per volume
V	volt
VSV	vesicular stomatitis virus
w/v	weight per volume
w/w	weight per weight

1. INTRODUCTION

1.1 General introduction

Measles continues to be a major cause of death among children in developing countries. The World Health Organization has estimated that up to 1.5 million deaths are caused by measles annually (Bart et al., 1983; Marusyk, 1984). Despite the availability of an efficient live attenuated virus vaccine measles has not been totally eradicated from western countries. An example of this is the large epidemics seen in Canada during 1985-1987. Measles virus (MV) normally causes an acute febrile illness followed by recovery and life-long immunity. However, in rare cases the infection becomes persistent causing a fatal progressing neurological disease in the central nervous system, subacute sclerosing panencephalitis (SSPE; ter Meulen and Hall, 1978).

Measles virus belongs to the Paramyxoviridae family, genus Morbillivirus. Other viruses included in the Morbillivirus genus are canine distemper virus (CDV), rinderpest virus of cattle, bovine paramyxovirus 107 and peste des petits ruminants virus of sheep and goats (Örvell and Norrby, 1985). A characteristic of the morbilliviruses is that there is no detectable neuraminidase activity and in attachment to host cell, the viruses use receptors insensitive to neuraminidase treatment. Another feature typical of morbilliviruses is the ability to form intranuclear inclusions in cytopathology (Norrby, 1985).

Measles virions are morphologically identical to other paramyxoviruses. They are pleomorphic, spherical enveloped viruses the diameter of which varies between 100 to 250 nm . The peplomers on the envelope project 9 to 15 nm from the surface (Norrby, 1985). The linear negative-strand RNA genome is included within the helical nucleocapsid and codes for six structural proteins and at least one nonstructural protein. The size of the MV genome has been estimated to be between 4.5-5.2 x 10^6 daltons (Baczko et al., 1983; Lund et al., 1984). It is associated with the large (L) and polymerase (P) proteins and surrounded

by a nucleoprotein (NP) capsid. Both the L and P proteins are involved in genomic replication but the replication events are not known in detail. The matrix (M) protein anchors the envelope containing hemagglutinin (H) and fusion (F) peplomers to the internal components of the virus. In addition, virions contain host cell-derived actin. (Tyrrell and Norrby, 1978). The nonstructural protein, recently named C protein, is not present in virions (Vainionpää et al., 1978). It is coded by the same gene than P protein but in a different reading frame (Bellini et al., 1985). This protein is believed to be a part of the replication process.

The gene order on the genome of MV has been found to be 3'-N-(P+C)-M-F-H-L-5' (Dowling et al., 1986; Rima et al., 1986). The genes coding for all the proteins except L have now been sequenced. Recently, Gerald et al. (1986) reported the nucleotide sequence of the hemagglutinin gene from the Hallé strain of measles and found that there is another reading frame in this gene which could code for a 70 amino acid protein. It is, however, not known at present time whether this protein is synthesized in MV infected cells.

1.2 Hemagglutinin

After the surface proteins H and F are synthesized and glycosylated in the MV infected cell, they are transported to the surface and inserted to the plasma membrane. Thus the final assembly of the virion occurs at the plasma membrane through which the virus buds. At this point, the virus receives its envelope which consists of the virus-coded surface proteins H and F and host-derived lipids.

Hemagglutinin is the surface glycoprotein of MV and is responsible for binding to the host cell whereas F protein mediates the entry into the cells (Norrby et al., 1984). Analogous proteins in other morbilliviruses do not have hemagglutination activity but are designated as H protein (Sheshberadaran, 1985). Paramyxoviruses other than

morbilliviruses display neuraminidase activity in the same protein and in these cases the protein is often designated as HN. The MV H protein is normally seen in gel electrophoresis as a band of approximately 79 Kd (Vainionpää et al., 1978; Varsanyi et al., 1984; Kramer and Cremer, 1984). However, the H peplomer is probably present on the envelope as a dimer (Lund and Salmi, 1981; Fujinami and Oldstone, 1981). The isolated H peplomers appear to form a truncated conical spike which is 6.5 nm to 4 nm wide and 22 nm long(Varsanyi et al., 1984). The glycosylation of H is not well characterized. The protein contains both simple and complex oligosaccharides (Bellini et al., 1983). By using external radiolabelling of the sugars, Anttonen et al. (1980) showed that the bond between the oligosaccharide and the protein backbone is alkali-stable which indicates N-glycosidic asparagine linkage. Anttonen et al. (1980) also showed that the carbohydrate chain has a terminal galactose residue and does not have sialic acid.

The amino acid sequence of the H protein was recently deduced from cloned mRNAs of both the Edmonston (Alkhatib et al., 1986) and Hallé strains (Gerald et al., 1986) of MV. The molecular size derived from the primary amino acid sequence is 69.25 Kd and the oligosaccharide part is approximately 10 Kd. Varsanyi et al. (1986) analyzed the N-terminal end of purified H. The amino acid sequence (residues 1-16) was found to be identical to the one predicted by Alkhatib et al. (1986) except that the initiator methionine is removed. A minor proportion of the mature H polypeptides started at position 3 (of the cDNA) which shows that H has a ragged N-terminal end. These results also confirmed that the N-terminal strongly hydrophobic part seems to anchor the molecule in the membrane. The predicted amino acid sequence shows five potential N-linked glycosylation sites between residues 168 and 240. It is not yet known which of these asparagines are actually glycosylated. Of interest is that a comparative analysis of the primary MV H sequence and paramyxovirus sequences gave no clue why MV H does not have neuraminidase activity (Alkhatib et al., 1986). Recent results suggest that MV has a low level neuraminidase

activity undetectable with previous techniques (Masellis, Marusyk and Armstrong, personal communication).

As mentioned above, the amino acid sequence of the H protein of Hallé has also been deduced from cloned cDNA. Despite the biological and antigenic differences normally seen between Edmonston and Hallé strains, the amino acid sequence homology showed a striking degree of conservation, 99.3%.

1.3 Antigenicity of hemagglutinin

The antigenicity of the H has been studied by several groups using monoclonal antibodies (MAbs). Ter Meulen et al. (1981) characterized the antigenicity of the H component of normal measles and SSPE virus isolates with 21 anti-H MAbs. Based on different tests including immunoprecipitation, hemagglutination inhibition (HI), hemolysis inhibition (HLI), neutralization (NT) and radioimmunoassay (RIA) they divided the MAbs into 5 functional groups with different properties. An important finding was that some of the MAbs had HI activity but did not neutralize some strains. Other MAbs could neutralize the virus but did not have HI activity. Their results suggested that SSPE and normal MV isolates have antigenic differences. However, no common pattern for the different groups could be found. To further characterize the relationships between the binding sites on the H protein, Carter et al. (1982) selected one representative Mab from each group for competitive RIAs. These antibodies formed three binding groups recognizing different but partially overlapping sites.

Giraudon and Wild (1985) used MAbs to study the correlation between epitopes of the H and biological activities. They defined four overlapping sites (A, B, C and D) by using a competition RIA. Sites A and B presented a high level of overlap and all the MAbs defining these sites had HI activity and neutralized virus infectivity. A second group of MAbs neutralized virus but did not have HI activity. This group corresponded to site D.

Only one of the MAbs failed to neutralize the virus and did not inhibit hemagglutination. This Mab defined site C. Giraudon and Wild (1985) also studied the modulative effect of these MAbs *in vivo* in a mouse model in which inoculated newborn mice normally die of acute encephalitis. The MAbs were inoculated at the same time as the virus and the disease was monitored. The antibodies formed three groups according to their ability to modify the disease. Only MAbs with HI activity could protect from the disease (functional group I) whereas some MAbs which neutralized *in vitro* but did not have HI acitivity could not prevent the disease (group II). One Mab induced a delayed neurological disease which was characterized by a low level of virus antigen synthesis (group III). These results demonstrated that there are at least three distinct domains in the MV H involving hemagglutination and neutralization. In addition, the observations suggest that the neutralization mechanisms *in vivo* may be different from those *in vitro*.

Sheshberadaran and Norrby (1986) characterized epitopes on the MV H using nine MAbs. In competitive binding assays these MAbs formed four partially overlapping binding groups. They also selected variants *in vitro* and analyzed these by radio-immunoprecipitation (RIPA), HI and NT assays. This way they could delineate seven operationally distinct sites. All the selected variants, however, possessed hemagglutination activity. Although there were some changes, none of the variants showed a significant decrease in reactivity with measles or SSPE sera in HI assays. In addition, all the variants retained neurotropic activity (90-100% mortality of newborn hamsters upon i.c. injection). Sheshberadaran and Norrby (1986) concluded that other immunodominant HI antibody generating sites must exist.

Other studies concerning the antigenicity of H have been made. Kramer and Cremer (1984) described a MAb against the H protein which had no activity in HI nor neutralization assays. This MAb seemed to recognize an epitope appearing late in infection (18-36 h) detected by immunofluorescence (IF), possibly reflecting a conformational alteration of the protein. Differences in a few epitopes between strains have been found

when a large number of MAbs have been used but most MAbs recognize equally well all the strains (ter Meulen et al., 1981; Trudgett et al. 1981; Sato et al., 1985; Bellini et al., 1986). Thus, the fact remains that measles as well as other paramyxoviruses are monotypic (Örvell and Norrby, 1985) with the exception of respiratory syncytial virus which has two biotypes. This is best seen in that, after a normal measles infection, a life-long immunity against the virus is acquired though subclinical reinfections can occur (Pedersen et al., 1986). Another indication of the monotypic nature of MV is that when antigenic variants induced by MAbs were selected *in vitro*, all variants retained most of their reactivity with human sera. Why paramyxoviruses are so different from orthomyxoviruses in this respect is not known.

No studies have been done thus far to localize antigen binding sites on the molecule. For example, most MAbs in these studies show neutralizing activity *in vitro* but the assumed attachment site for the cell receptor remains totally unknown. The domains including the hemagglutinating activity have not been characterized. Approaches that could be used for studying these would include, for example, expression of parts of the gene in a system where the fragments could be isolated. Synthetic peptides can also be used for this purpose but thus far neither of these approaches have been used.

1.4 Fusion protein

The other peplomer present in the measles virion envelope is the F protein which is responsible for fusion of cytoplasmic membranes and lysis of erythrocytes. The fusion protein is synthesized in MV infected cells as a precursor protein designated as F_0. The virions are not fully infectious unless F_0 is cleaved by host derived enzymes to two components, F_1 and F_2 (Fujinami and Oldstone, 1981, Norrby et al., 1984). These components are then connected by a disulfide-bond. The molecular weight of the F protein under nonreducing conditions has been reported to be 64 Kd (Fujinami and Oldstone,

1981). Under reducing conditions the F_1 and F_2 components are separated and migrate at 41 Kd (Varsanyi et al., 1985) and 18-22 Kd (Norrby et al, 1984), respectively. Only F_2 is glycosylated. When F peplomers are isolated from virions, they have a club-like shape (dimensions of the oval head 6 x 9 nm and length 16 nm) in electron microscopic examination at pH 6.0 (Varsanyi et al., 1984).

Recently, Richardson et al. (1986) cloned the messenger RNA corresponding to the F protein of MV and sequenced the resulting cDNA. Only one open reading frame capable of coding for 550 amino acids was found. The molecular mass of the predicted protein is 59.51 Kd before post-translational processing. It contains two potential glycosylation sites both of which are on F_2. The predicted F_1 amino terminus was found to be identical to the sequence determined by Varsanyi et al. (1985) by direct protein sequencing. Richardson et al. (1986) also predicted the secondary structure. This showed that F_2 is likely to form a globular head which could be involved in mediating of the entry of the virion to cells. The predicted secondary structure of the F protein revealed similarities to the functional domains of the influenza hemagglutinin protein.

At the amino acid level, relatively little homology was observed when compared to simian virus 5 (SV5) or Sendai virus and even less compared to respiratory syncytial virus (RSV). However, in F_1 the terminus 9 cysteine residues are conserved as well as 5 proline residues situated at similar locations. A well established fact is that disulfide bonds require cysteine whereas proline residues are important in forming turns. Thus, conservation of these residues can result in very similar folding of the F proteins of different paramyxoviruses. In addition, the distribution of hydrophobic residues had apparent similarity in all these viruses and the authors suggested that this distribution would determine the fusion activity. Richardson showed that oligopeptides with amino acid sequence similar to the N-terminus of F_1 protein of most paramyxoviruses could inhibit the infection and hemolysis induced by MV as well (Richardson et al.,1980; Richardson and Choppin, 1983). This also supports the idea that the fusion activity carried by different

paramyxoviruses is located in similar positions, probably close to the amino terminal region of F_1 . Recently, Portner et al. (1987) selected three antigenic variants of Sendai virus with an anti-F MAb which was able to inhibit hemolysis and also had neutralizing activity. Sequence analysis of the F gene of these mutants showed a single mutation which seemed to be abolish the reactivity of the MAb. This mutation was at residue 399 which is far from the amino terminus of F_1. A synthetic peptide derived from the sequence of this region was recognized by the MAb indicating that the site of mutation actually is the site of binding. Portner et al. (1987) suggested that the three-dimensional structure must be folded in a way which brings the residues around position 399 to close proximity of the amino-terminus of F_1. As yet, there is no knowledge of the three-dimensional structure of any of the paramyxovirus surface proteins.

1.5 Antigenicity of fusion protein

Relatively few studies concerning the antigenicity of MV F protein have been done largely because of the difficulties in purification. However, MAbs have provided a means to study the F antigenicity. Sheshberadaran raised MAbs against the F protein and characterized these using several assays including NT, HLI, radio-immunoprecipitation (RIPA) and Western blot (Sheshberadaran et al.,1983; Sheshberadaran, 1986; Sheshberadaran and Norrby, 1986). Epitope characterization based on results of these tests delineated at least six antigenic sites for F protein. However, in competitive assays these MAbs formed a maximum of four sites (Mäkelä and Salmi, unpublished results). None of the MAbs neutralized virus and only low HLI titers were observed. In Western blot, none of the antibodies reacted with the antigen indicating that the MAbs are against strongly conformation-dependent sites.

Shesheberadaran tested the anti-F MAbs against nine different lytic strains of MV (Shesheberadaran et al., 1983; Sheshberadaran, 1985). No variation in reactivity of the

MAbs with the different strains was found which shows the conserved nature of the F component. However, study of several persistently infected cell lines revealed large differences in the antigenicity of the F protein (Sheshberadaran et al., 1985).

Sheshberadaran and Norrby (1984) found that three MAbs against F cross-reacted with cellular stress proteins. Stress proteins are synthesized in cells only under 'stress', for example, heat shock and possibly even virus infection. This could be a factor playing a role in virus-induced autoimmunity. As other viruses and physico-chemical factors are capable of inducing stress proteins (Sheshberadaran, 1985), these entities could predispose to a boosting effect caused by MV as the stress proteins would have previously been seen by the immune system. However, there is no *in vivo* evidence to support this theory as antibodies against these proteins have not been found in human sera in measles infections (Örvell and Norrby, 1985).

Varsanyi et al. (1984) purified F protein by affinity chromatography and showed by polyacrylamide gel electrophoresis that the preparation was free of cellular or other virus-specific proteins. They raised polyclonal hyperimmune serum and used RIPA to demonstrate the specificity to F protein. This serum had a high HLI activity, did not react with the H and could neutralize virus infectivity. This was the first demonstration that MV can be neutralized by antibodies against F protein. This is in accordance with Merz et al. (1980) who showed that antibodies against F are important in preventing the spread of infection of paramyxoviruses. Norrby et al. (1984) have also shown that polyclonal anti-F serum prevents cell-to-cell transmission of measles.

To further characterize the importance of the surface proteins of paramyxoviruses in eliciting immune defense, Norrby et al. (1986) immunized dogs with purified F and H proteins of CDV. None of the dogs immunized with F protein showed any clinical signs after exposure to live virus whereas two out of three dogs given H antigen showed symptoms. Löve et al. (1986) showed that a MAb to the F protein of mumps virus protected newborn hamsters from necrotizing mumps meningoencephalitis. Using

recombinant vaccinia viruses, Paterson et al. (1987) recently showed that both HN and F proteins of SV5 could protect hamsters from challenge when infected with SV5. All these studies demonstrate that the F protein of paramyxoviruses is important in inducing immune protection and implies that in design of synthetic vaccines for morbilliviruses, this component should be included.

1.6 Measles virus persistency

The mechanisms leading to a persistent infection are still poorly understood . In most cases, SSPE occurs in individuals who have suffered uncomplicated measles at less than two years of age (Wechsler and Meissner, 1982). The virus obviously reaches the central nervous system (CNS) at some time during the acute measles and establishes a persistent infection. Fournier et al. (1985) demonstrated, using *in situ* hybridization, that a high proportion of both circulating lymphocytes and perivascular cuffs contain MV specific RNA sequences. This suggests that infected lymphocytes could provide a transportation mechanism for the virus to enter the CNS. Another alternative mechanism is that during the transient immunosuppression caused by the acute infection this neurotropic virus may enter the CNS unchallenged by the immune system (Griffith, 1985). How the virus is then able to remain in the body and escape host immune surveillance is not understood. However, the persistent infection results in development of fatal progressing encephalitis one to ten years after the primary infection. Infectious virus can only be isolated from diseased brain by cocultivation techniques as few, if any, budding virions are formed (Baczko et al., 1986, Cattaneo et al., 1986).

Several theories of mechanisms involved in establishment of persistency have been described. Joseph and Oldstone (1975) and Fujinami and Oldstone (1980) studied the effect of antibodies on virally infected cells. It was found that expression of viral gene products on the surface of these cells changes when they are incubated with antiserum

against MV. This phenomenon, called antibody-induced antigenic modulation, offers a theory for the mechanism of pathogenesis of SSPE. As has been pointed out above, SSPE occurs often in individuals who had measles at less than two years of age. It may be that these children still have some maternal antibodies against measles left at the time of infection which are, however, not abundant enough to prevent the infection. Modulation of the expression of the viral proteins may create a new, mainly cell-associated, variant of virus which does not induce a vigorous immune response against the infected cell and is thus able to survive the host defense. To further support this theory, it has been recently shown that even MAbs against certain epitopes on the MV H protein can change the expression of other structural polypeptides in a cell culture (Fujinami et al., 1984).

Rammohan et al. (1982) observed a case in which a two-year old child was given immune serum globulin after her older sister contracted measles. Despite the passive prevention, normal measles followed within a week. However, five years later the girl developed SSPE. This observation led Rammohan et al. (1982) to study the role of antibody in viral persistence. When they infected four to six weeks old Balb/c mice with the hamster neurotropic measles virus, the animals developed acute encephalopathy which was fatal in 80% of the animals. Administration of antibody against measles three days after the virus inoculation completely abolished the acute disease but the mice developed a delayed encephalitis with onset varying from four to eight weeks. It is possible that extracellular neutralization of MV limited the spread and thus delayed the onset of encephalitis. However, viral antigen expression was persistent and also the onset of the disease did not correlate with disappearance of the passively administered antibody. Thus, antigenic modulation could have happened *in vivo* resulting in formation of a cell-asssociated strain of MV causing the modulated disease. The final mechanisms leading to destruction of the brain are, however, not known. It may be, as Carrigan has suggested (1986), that this is a direct result of accumulation of defective virus causing the damage.

Two other reports on antigenic modulation employing MAb to the H *in vivo* have been published with similar results (Rammohan et al., 1981; Rammohan et al., 1983).

Carrigan (1985; 1986a; 1986b; Carrigan and Kabacoff, 1987a, b) has postulated a theory based on experiments done with newborn hamsters. In this model, when the LEC strain of MV is inoculated in to brains of newborn hamsters, a normal productive infection is first seen. In a few days, however, the productive infection converts to a cell-associated state and the amount of infectious virus is strongly reduced. At this stage, the number of infected brain cells is very low and the virus can only be isolated with co-cultivation techniques. Carrigan proposed a hypothetical course of the experimental disease which can be applied to SSPE as well. In the first step, the CNS is infected with the virus which could be heterogeneic or become heterogeneic during the initial replication of the virus. Thus there would be two biologically different virus populations present in the brain, early in infection, one of which would replicate productively and the other would be a cell-associated nonprogressive virus. In the second phase, only a few days after initial infection of the CNS, the replication and spread of the productive form of the virus is limited in the brain by immunological mechanisms. Neutralizing antibodies have not yet been formed at this stage so these mechanisms may be at least partly nonspecific involving, for example, interferon (Carrigan and Kabacoff, 1987a,b). At the third stage, specific immunity, eg., cytotoxic T cells, eliminates the remaining productively infected cells. After this, only the cell-associated virus resistant to host defense, is left in the infected host. This form of virus can then slowly spread and the resulting damage accumulate in the brain.

Hall et al. (1979a) found that SSPE patients do not have antibodies against the M protein despite of the hyperimmune state against other proteins (Salmi et al., 1972). This led to several investigations of the expression of M protein both in persistently infected cell cultures (Carter et al., 1983; Sheppard et al., 1985; Young et al., 1985) and in brains of SSPE patients (Hall and Choppin, 1981; Baczko et al., 1984). Most of these studies have shown defects in expression of matrix protein in SSPE patients. Haase et al. (1985) used *in*

situ hybridization and immunoperoxidase techniques to study the expression of the genome in brain tissue obtained after the onset of SSPE and in the terminal phase. They showed that early in the disease the expression of the whole genome is repressed and very little if any antigens are synthesized. Late in the terminal phase they found accumulation of nucleoprotein but still no M protein, in accordance with the earlier studies. Cattaneo et al. (1986) reported that normal monocistronic mRNA in SSPE brain was substituted by a bicistronic RNA which contained the coding sequences of both M and P proteins. They also found in this case a point mutation leading to a stop codon in the beginning of the M gene which would explain why no M protein was translated.

Recently, however, Norrby et al. (1985) were able to detect M protein in brains of four victims of SSPE by IF using MAbs. Baczko et al. (1986) also found in one of four patients M protein expressed using the same technique. By isolating the virus-specific mRNA and translating it *in vitro*, M protein was synthesized from the RNA derived from the same brain in which M protein was detected with fluorescence. However, in this study the amounts of F and H proteins varied between the patients and the expression of these proteins was deficient . Similar findings have been obtained in cell cultures infected with hamster neurotropic and LEC strains of MV (Löve et al., 1986). This leads to the question whether the lack of M protein is the only reason for defective assembly of the virions or if defects in other genes caused by cellular restriction might be partially responsible for this phenomenon.

Thus far, none of the postulated mechanisms of persistency has been proven to be more valid than any others. It is obvious that we have to understand better the host-cell interactions. To do this, other factors, including the immune response elicited by the virus, affecting this complex system have to be studied at a more detailed level.

1.7 Humoral immune response to MV

In normal MV infection, IgM class antibodies are detected in serum shortly after the appearance of rash. The peak response is seen within 10 days after the onset of the rash and the titers start to decline thereafter reaching undetectable limit two to three months later. (Vuorimaa et al.,1978). IgG class antibodies increase in titer at the same time but these remain at high levels for years. The antibody levels decrease slightly over time but can persist over a lifetime to guarantee protective immunity as shown by Panum (cited by Krugman et al., 1965).

In SSPE, a highly accentuated humoral immune response has been found in many studies (Connolly et al., 1967; Salmi et al., 1972; Hankins and Black, 1986). The difference of antibody titers between sera from SSPE patients and normal late convalescent sera is 10-100 fold (Norrby et al., 1981). This response is mainly of the IgG class but recently two studies have been published in which IgM class antibodies were found both in serum and CSF of at least some SSPE patients (Chiodi et al., 1986; Ziola et al., 1986). This, however, seems not to be a marker of the onset of the disease as the frequency of the presence of these antibodies is low even during the first year of the disease (Ziola et al., 1986).

Atypical measles is another form of disease which is characterized by high antibody titers to MV (Norrby et al., 1981). Atypical measles presents with more severe symptoms than regular measles including edema, hemorrhage and abdominal pain in addition to the normal signs. This disease developed relatively frequently in individuals who had received killed MV vaccine. This was shown by Norrby et al. (1975) to be due to the absence of detectable antibodies against F protein after vaccination with the inactivated vaccine. It has later been shown that antibodies to the F protein of paramyxoviruses in general are necessary to prevent spread of infection (Mertz et al., 1980). The pathophysiology leading to atypical measles, however, is not known. It has been suggested that the syndrome is

caused by the absence of humoral response to F protein combined with a hyperactive lymphocyte proliferative response resulting in immune imbalance leading to the disease (Krause et al., 1978).

Patients with multiple sclerosis (MS) also have increased titers to MV (Adams and Imagawa, 1962) both in serum and cerebrospinal fluid (CSF; Salmi et al, 1972). The difference compared to normal blood donors, however, is not as striking as in SSPE and atypical measles. MV is, because of these findings, often linked to MS as one of the etiological factors. However, it should be noted that the elevation of viral antibody level is not unique to MV. Intrathecal antibody synthesis against other common viruses including rubella, parainfluenza 2, mumps and influenza has been detected and could, therefore, reflect a general phenomenon involving immunoregulatory deficiency (Salmi et al., 1983).

The antibody level to MV is elevated in other diseases such as systemic lupus erythematosus (SLE; Laitinen and Vaheri, 1974), rheumatoid arthritis (Kalliomäki et al., 1975) and chronic active hepatitis (CAH; Shirodaria et al., 1985). A case has also been reported in which a patient suffering from mental retardation and seizures 24 years after measles encephalitis had a highly increased humoral response to MV (Hall et al., 1979b). Whether these findings are relevant to the pathogenesis of these diseases is not known. However, as all of these diseases seem to involve a general immunoregulatory disturbance, it could be speculated that the elevation of MV antibodies merely reflects a humoral imbalance in these cases.

1.8 Polypeptide-specific antibody response to MV

Commonly used methods for evaluating component specific antibodies to MV include complement fixation (CF), HI and HLI tests which measure mainly antibodies to nucleocapsid (NC), H and F proteins, respectively. Norrby and Gollmar (1972) used these tests to analyze the appearance and persistence of antibodies to different components after a

normal MV infection. Antibody titers in acute, early and late convalescent sera were measured. Generally, HI and HLI titers correlated well with each other although in few patients a high HLI titer in the presence of low HI titer was seen. The results also demonstrated that some HI antibodies can block lysis of red cells which shows that the HLI test is not specific to the hemolysin component of the virus (F protein). The CF test was performed with whole virus material, purified nucleocapsid (NC) and purified small particle H , prepared by trypsinizing purified virus and isolating the H component. In all sera, antibodies detected against the nucleocapsid dominated whereas hemagglutination (HA) titers measured with CF were very low. Norrby and Gollmar (1972) also used gel diffusion and found anti-NC antibodies dominating as well. Norrby and Gollmar (1975) later showed that hemolysin-specific antibodies can be measured after absorbing the serum with Tween-80 treated MV material.

As implied earlier, none of the tests mentioned are very sensitive or specific. Modern techniques, therefore, must be used to analyze polypeptide-specific response to MV. Hall et al. (1979a) used RIPA to study the antibody response of SSPE patients. It was found that there is little antibody present against M protein in sera from SSPE patients although atypical measles sera showed relatively good reactivity to M protein. Trudgett et al. (1980) used a solid-phase RIA to measure antibodies from acute convalescent and normal convalescent sera and from sera of SSPE patients. They found antibodies against all the major polypeptides in SSPE sera. Qualitatively, all sera were similar and contained antibodies reacting with polypeptides of both a SSPE strain (Mantooth) and vaccine strain (Edmonston) MV. The assay involved electrophoresing the MV antigen followed by overlaying of the gel with the serum. The results suggested that acute convalescent and SSPE sera had higher antibody levels than normal convalescent sera. In addition, variation in reactivity with the individual polypeptides was found in each serum group.

Machamer et al. (1980) analyzed acute, early and late convalescent sera from patients with natural measles infection and from patients vaccinated with a live attenuated

virus, sera from patients with atypical measles and sera and CSF from measles encephalitis patients. They used RIPA to determine the polypeptide-specificities of the antibodies present in the sera and CSF. Neither acute sera nor CSF of encephalitis patients had detectable antibodies whereas both early and late convalescent sera had qualitatively similar patterns precipitating mainly H, F, NP and P proteins. Matrix protein was precipitated efficiently only by the atypical measles sera although also six out of eight early convalescent sera precipitated M protein at low dilutions. An attempt to quantitate the amount of antibody was done by using serial dilution and determining the relative amount of precipitation by photometric scanning and integrating the peaks obtained. Of interest is that atypical measles sera M-specific antibody titers were clearly the highest. There seemed to be a correlation between the clinically apparent symptoms of measles and anti-M antibodies, i.e. early convalescent sera had higher titers than analogous sera after vaccination. The titers against the surface proteins were generally equal within all groups and again, atypical measles sera had the highest titers compared to the other sera. Unexpectedly, neutralizing activity did not correlate well with the ability to precipitate H and F proteins. For example, one natural infection late convalescent serum had the same neutralizing titer as a late convalescent serum after immunization, yet the former better precipitated both H and F proteins. It was noted, however, that the sites against which antibodies are measured in NT and RIPA tests may be different which would explain the discrepancy.

Norrby et al. (1981) used RIPA and CF test employing purified M and NP antigens to measure antibodies against M and NP in different disease conditions. The sera analyzed included early and late measles convalescent sera and sera from patients with MS, SSPE, CAH and atypical measles. Generally speaking, all the sera tested had a high ratio of anti-NP/M titers in RIPA with the exception of atypical measles. CF test involving purified M protein seemed to be more sensitive than RIPA in detecting antibodies to M but titers compared to those against NP were low. MS patients in this study did not have higher

antibody titers than late convalescent sera. Atypical measles patients had high anti-M titers. This was speculated by the authors to be caused by the immunopathological reaction enhancing the total immune response. An alternative is that for some reason the sensitization with the killed vaccine against M is enhanced.

Graves et al. (1984) also employed immunoprecipitation to study the appearance and frequency of antibody responses to different polypeptides after natural measles. According to this study, antibodies to NP develop first and are present at the onset of the rash. They are also predominant antibodies made which is in accordance with the results of Norrby et al.(1981). Antibodies to H and F proteins were present in about 80% of patients at the onset of rash. However, the response against H was higher than to F. Confirming earlier reports, antibodies to M protein were, in general, detected only at low levels and many individuals had no antibodies.

Hankins and Black (1986) reported contradictory results in the antibody response in MS, SSPE, atypical measles patients and in normal persons using Western blot. Firstly, antibody to M protein was detected in relatively normal levels in SSPE patients. Most earlier reports, including the first by Hall et al. (1979), have used immunoprecipitation for detecting antibody to M. However, it was demonstrated by Ohara et al. (1985) that buffers used for immunoprecipitation seem to be critical in the tests for M protein. Thus, Western blots can be a more suitable method for detection of antibody to M. MS patients, however, had reduced levels of antibody to M protein although levels to other proteins were comparable to normal late convalescent patients. This is in accordance with the report by Hayes et al. (1980) who found antibodies to all virus antigens except M in MS sera.

A large increase in antibodies against P was seen from early to late convalescent sera. This is controversial as there should not be antigenic stimulus long after the infection, yet the antibodies increase. Wechsler and Meissner (1983) previously found that 5 out of 24 MS patients had higher levels of anti-P antibodies than normal sera. In addition, Wechsler et al. (1979) found in their earlier study that antibodies to P dropped from early to

late convalescent sera contradictory to the results of Hankins and Black (1986). However, antigenic determinants against which antibodies are measured in RIPA and Western blot, are different which could explain the differences in the results obtained in these two studies. Hankins and Black (1986) also observed differences between the groups in anti-H antibodies. Acute and late atypical measles sera had lower relative levels of anti-H than late convalescent sera. This could have implications in the pathogenesis of atypical measles as the anti-H response is essential for neutralization of the virus.

To summarize the polypeptide-specific response to MV, it is probably justified to say that better methods to quantitate antibodies to different components are warranted. Many reports may be contradictory because different antigens behave differently in different assays. It is therefore difficult to compare the results obtained by various methods.

1.9 Cell mediated immunity to MV

Cell mediated immunity has a crucial role in recovery of the primary disease of measles as agammaglobulinemia patients can clear the infection in a normal manner (Good et al., 1962). During the convalescent acute phase of measles, cellular immunity can be demonstrated with the T cell proliferation assay (Ilonen et al., 1980a; Greenstein and McFarland, 1983) and cytotoxicity assay (Sissons et al., 1985). A peculiar fact, however, is that both of these activities decline over a period of weeks. The majority (up to 95%) of seropositive normal adults have very low cell mediated responses as detected by a T cell proliferation assay (McFarland and McFarlin, 1979; Ilonen et al., 1980b; Greenstein and McFarland, 1983) or T cell killing of MV-infected target cells (Greenstein and McFarland, 1982; Lucas et al., 1982; de Hullu, M.J., personal communication).

A widely studied phenomenon associated with the cellular response is the transient immunosuppression during measles infection. This occurs both *in vivo* (Hirsch et al.,

1984) and *in vitro* (Sullivan et al., 1975; Borysiewicz et al., 1985). The mechanisms are poorly understood but infection of different lymphoid cell populations by the virus may play a role (Sullivan et al., 1975; Huddlestone et al., 1980).

Although several studies on cell mediated immunity in measles have been done, the role of different components of the cellular arm of the immune system is poorly understood. For example, MV-specific cytotoxic lymphocytes (CTL) have been demonstrated in acute measles but seem not to be HLA class I restricted (Sissons et al., 1985). Instead, HLA class II restricted clones have been generated from MS patients (Jacobson et al., 1984). These findings are supported by comigration experiments in which no association between surface proteins of MV and HLA I antigens was found (Oldstone et al., 1983). However, the frequency with which MV-specific CTLs can be demostrated seems to be low. In MS, the activity of CTLs seems to be even lower than in normal persons (Jacobson et al., 1985).

Few reports are available concerning the T cell reactivity of different components of MV. This is largely because of problems in preparing the polypeptides to the necessary purity. Ilonen et al. (1980 b) used purified MV virions, plasma membranes isolated from MV infected cells and purified nucleocapsids as stimulating antigens in a lymphoproliferative assay. Peripheral blood mononuclear cells were isolated from 38 normal seropositive controls and four SSPE patients and stimulated with the antigens. All controls and three of the SSPE patients had weak or no response to any of the antigens. However, one SSPE patient responded at one time of the disease course to the virus. It was found that isolated plasma membranes were equal to purified virus in stimulating the lymphocytes and also NP elicited a response although at a lower level. Bellini et al. (1981) studied lymphoproliferative responses of three measles high-responders (HR) and two low-responders (LR) to H purified by affinity chromatography. One of the HRs responded equally well to pure H and a MV infected plasma membrane preparation and the other two HRs also had good reactivity to H though less than to membranes. These results suggested

that the H protein is one of the major antigens against which proliferative response is directed. Rose et al. (1984) further analyzed the reactivity of all the major antigens of MV in a proliferative assay. The antigens used were purified from virions by PAGE and eluting the fractions from the gel. The greatest responses were against HA and F_1 supporting the earlier finding that the H protein was one of the major antigens in this assay. However, relatively good responses were also observed to P, M and NP proteins. Richert et al. (1986) generated MV specific T cell lines and clones from a MS patient and analyzed the antigen specificity of each. Three of the clones reacted with F, one with H and two with MV but not with purified proteins. The five cell lines had multiple specificities indicating that they were not sufficiently subcloned . However, all lines responded to H and F whereas only two reacted with M and two with NP. These results, though based on one patient only, again emphasize the role of surface proteins in the proliferative T cell assay. This far, no studies showing polypeptide-specificity of CTLs against MV have been available.

1.10 Immunization against measles

Measles was isolated for the first time by Enders and Peebles in 1954 in human renal cells (Mitchell and Balfour, 1985). This was followed by adaptation of the virus to different cell lines and finally development of the live attenuated vaccine. The first vaccine brought onto the market in the United States in 1963 contained the Edmonston B strain and two others were later introduced, Schwartz in 1965 and Moraten in 1968. At the time when live attenuated vaccines were developed there was some concern about possible adverse effects, especially vaccine-induced encephalitis. Consequently, a killed formalin-inactivated vaccine was made and used in parallel with the live attenuated preparation. It became apparent by the late 1960's that the killed vaccine did not offer sufficient protection and, more importantly, often led to development of atypical measles. As has been

discussed earlier, the killed vaccine elicited a qualitatively different immune response than the live vaccine. Firstly, the cellular immunity seemed to be hyper-reactive to MV and secondly, the humoral response lacked antibodies to F protein, as shown by Norrby et al. (1975). The killed vaccine was not used during the 1970's any more and it has been recommended that all recipients be revaccinated with live attenuated MV as the expected encephalitis rates are much lower than estimated. The antibody response after vaccination with the live vaccine has been reported to be similar to the one elicited by natural infection although the titers seem to be somewhat lower than after natural infection (Machamer et al., 1980; Mitchell and Balfour, 1985; Hankins and Black, 1986). Studies have been done on the persistence of the antibody levels. Krugman et al. (1965) showed that although a small decline was observed, HI titers remained at relatively high levels for at least 4 years after administration of Edmonston B vaccine. In recent years, the most commonly used vaccine in North America has been a combined measles-mumps-rubella vaccine. It has been shown that antibodies to all components of this vaccine persist for at least seven years after administration (Weibel et al., 1979).

Pedersen et al. (1986) studied the antibody response after vaccination in an isolated community in Greenland . This society had not been previously exposed to measles and the immune status was followed for 16 years after vaccination with the Schwartz strain of more than 90% of the people in the community (approximately 500). IgA, IgM and IgG EIA assays and the HI test were used to measure serial samples from 55 persons. Vaccination caused seroconversion in 94-100% of those tested. After 16 years, 43% had HI titers higher than 10 and 70% had detectable IgG antibodies. During this time, no measles cases were reported. However, in some individuals a rise in antibody titers was seen 2-4 years after immunization indicating an assumed reinfection by natural MV although no clinical signs were seen. Thus it is possible that a communal boost of the antibodies in the population had occurred. Even taking into account the contributing effect of natural boosting, it would seem that in this population the number of susceptible persons

is increasing. It has to be noted, however, that the Schwartz strain has been claimed to elicit a lower response than Edmonston B vaccine or natural infection.

The vaccination programs in western countries have proven to be highly efficient. In the United States, the rate of measles and its complications has declined more than 99% since the prevaccine era (Levy, 1984; Bloch et al., 1985; Mitchell and Balfour, 1985). This has to be credited to an efficient immunization program (>90% of young susceptibles) but a contibuting factor is that 99% of the adult population is naturally immune to measles. Taken together these numbers have decreased the susceptible population so that few epidemics are seen at present. However, as natural immunity wanes and the majority of the population have protection induced by vaccination only, the number of susceptible people increases. This happens firstly because it is difficult to attain 100% vaccination rates and secondly, the seroconversion rate is not 100%. Based on these facts Levy (1984) calculated that though the proportion of susceptibles in the United States was as low as 3.1% in 1978, it has increased since 1981 by roughly 0.1% per year and will reach 10.9% of total population by 2050. This is based on the assumption that the global immunization programs will fail, as many such programs have this far, and there is virus present to incite the disease. This theoretical approach is actually supported by recent reports on measles outbreaks in high schools and universities (Gustafson et al., 1987; Nkowane et al., 1987). In 1986, the incidence of measles was 2.7 per 100,000 population compared to 1.2 in 1985 (MMWR, 36(20), 1987). There have been relatively large epidemics in 1986-1987 in Canada as well. The striking feature about the epidemics is that the effected age groups are becoming older (Marusyk, 1984).

In developing countries there are other problems with vaccination in addition to those in western countries. Heat-lability of the live vaccines is a constant problem in areas where cold-handling is rarely available. This has been one of the major reasons for vaccination program failures in developing countries.

Taken together, it is obvious that we do not yet have an ideal vaccine for measles virus and we are far from total eradication of measles. Thus, a detailed study of the immunogenicity of the virus is warranted.

1.11 Use of synthetic peptides in studies of antigenicity and immunogenicity of viral proteins

One approach to study the antigenicity of viral proteins is to chemically synthesize peptides from known amino acid sequences of the polypeptides. This methodology has progressed rapidly since the beginning of 1980's because of the potential use of synthetic peptides as vaccines. However, the idea of producing antibodies against short peptides was proposed in 1963 by Anderer (cited by Brown, 1984). A peptide was digested from the C-terminus of tobacco mosaic virus coat protein and antibodies were raised against it. The antibodies could bind to the intact virus and neutralize infectivity. After a silent period of about 15 years, several encouraging reports about the ability of synthetic peptides to elicit antibodies recognizing native structures were published (Lerner et al., 1981; Prince et al., 1982; Bittle et al., 1982; Dreesman et al., 1982; Emini et al., 1983). Based on these studies it became obvious that the capability of short peptides to generate protein-reactive antibodies was actually more general than was assumed (Niman et al., 1983) . This also changed the previous concept that antigenicity and immunogenicity are equivalent. It is now generally agreed that practically the whole surface of a protein is immunogenic although only some sites can dominate in the natural immune response (Lerner, 1982; Westhof et al., 1984).

The advantage of synthetic peptides is that the generated antibodies have a well-determined specificity. Thus they can be applied as tools in localization of neutralizing sites (Elder et al. 1987), characterization of gene products, purification of proteins, detection of proteins in diagnostic assays, studies of protein function etc. (reviewed by Walter, 1986).

However, prediction of the sites to be synthesised presents a problem. In an ideal case, the amino acid sequence can be combined with three-dimensional structure for mapping antigenic sites. Crystallography is the only method thus far which can be used for determining thrtee-dimensional structures. As it is extremely laborious, expensive and time-consuming method it can not be widely applied. Therefore, other methods have to be used. The most common parameter for predicting surface exposed regions which might be antigenic sites is hyrophilicity-phobicity (Hopp and Woods, 1981). The number of hydrophilic regions on a large protein is often clearly greater than the immunodominant 'real' sites. As a result, more parameters are needed for practical purposes when the sites are selected. Prediction of the secondary structure has been used for analyzing the amino acid sequences of proteins. There is no convincing evidence, however, that adding of secondary structure prediction to hydrophilicity profiles would improve the chances of finding antigenic sites. Indeed, Walter (1986) concluded that, for all practical purposes, prediction of secondary structure does not offer any advantage over using only hydrophilicity profiles.

Westhof et al. (1984) used yet another parameter to correlate antigenic sites. They showed that with temperature factors (B values), which represent mean-square displacement of each atom, it is possible to produce an image of the segmental mobility of the polypeptide backbone. This analysis was found to predict antigenic sites of lysozyme, myoglobin and tobacco mosaic virus protein better than hydrophilicity. Parker et al. (1986) generated a new set of hydrophilicity parameters and combined these with accessibility (Janin,1979) and flexibility (Karplus and Schultz, 1985) parameters. By creating composite profiles of different proteins with these three sets of parameters, they correlated known antigenic sites to the predicted ones. The composite profile seemed to correlate well with the X-ray-determined sites of influenza hemagglutinin.

Despite the rapidly developing methods for prediction of antigenic sites derived from primary amino acid sequence, the success in possible vaccine development with

synthetic peptides in virology has not been very good. At the moment, the studies furthest advanced in using synthetic peptides have been done with hepatitits B, polio, foot-and-mouth disease and influenza viruses (Steward and Howard, 1987). Bittle et al. (1982) were succesful in their experiments with synthetic peptides corresponding to regions of the VP1 protein of foot-and-mouth disease. One of the peptides, residues 141-160, elicited virus neutralizing antibodies in guinea pigs after single inoculation and protected the animals from subsequent challenge with live virus. This region was serotype-specific but obviously the other six serotypes have immunogenic activity in this region as well (Rowlands et al., 1983). Of interest is that purified VP1 protein has a very low immunization activity (Bittle et al., 1982; Steward and Howard, 1987).

Similar results have been obtained with poliovirus type 1 using synthetic peptides derived from the sequence of VP1 . In a study by Emini et al. (1983), all five peptides synthesized could prime for serotype-specific neutralizing antibodies when the rabbits were inoculated with intact virus. This was the first demonstration of the priming effect with synthetic peptides. This could be used in the future as a vaccination strategy, i.e. when it is preferrable to induce a response to selected antigens, peptides could be used for priming or boosting. Studies with influenza virus have led to another idea for the use of peptides as vaccines. As has been pointed out earlier, the whole surface of a protein can be considered immunogenic (Green et al., 1982). Müller et al. (1982) synthesized a peptide which has not been reported to be part of the proposed antigenic sites of influenza hemagglutinin. The antibodies elicited against it recognized intact hemagglutinin and could cross-react with another subtype of virus. If regions possessing sufficiently high immunogenic activity were found in conserved areas of the H molecule, these could be included in a vaccine to induce a response against several subtypes.

Hepatitis B is a good example of strongly progressing research area in peptide vaccine development. Numerous encouraging reports describing the immunogenicity of peptides derived from the sequence of the surface antigen have been published (Prince et

al., 1982; Dreesman et al., 1982; Gerin et al., 1983; Thanavala, 1986). Recently it has been found that pre-S domains of the *env*-protein are responsible for attachment of the virus to hepatocytes. Neurath et al. (1986) have synthesized peptides corresponding to the pre-S sequences of the *env* middle protein. This area seems to be highly immunogenic and the peptides from this region bind significantly antibodies from sera of human carriers.

Most studies done thus far in probing the antigenicity of viral proteins with synthetic peptides have only dealt with B cell recognition. However, as it has become apparent that cell-mediated immunity has probably as big a role as humoral immunity in clearance and prevention of viral infections, increasing emphasis is now directed to T cell recognition of viral proteins. It is still a matter of controversy how much overlapping of T and B cell determinants on viral proteins there is. Atassi and Kurisaki (1984) introduced comprehensive synthetic surface scanning for localization of antigenic determinants of influenza hemagglutinin. In their study, 12 synthetic peptides were highly immunogenic in producing antibodies and of these, 6-7 peptides stimulated *in vitro* blastogenic response to virus-primed T cells. This suggests that the determinants are at least partially overlapping. Milich et al. (1986) presented contradictory results with pre-S region determinants of hepatitis B surface antigen. In their study, none of peptides previously shown to define an important B cell determinant were recognized by T cells. However, only three peptides of one major region were used in this study which does not rule out that other B cell determinants might be recognized by T cells. Studies with influenza and herpes simplex viruses and with cytochrome C would also support Atassi's view of partially shared determinants (Hackett et al., 1985; Watari et al., 1987; Kilgannon et al., 1986).

Briefly, synthetic peptides do provide feasible tools for analysis of the antigenicity of viral proteins. The previous concept that short peptides can only elicit antibodies which recognize linear continuous determinants has proven to be wrong. Therefore, hope of using peptides at least as part of component vaccines is still realistic. However, with the

prediction methodology used thus far for selecting regions to be synthesized, it seems to be more or less fortuitous to actually find biologically important determinants of viral proteins.

2. AIMS OF THE STUDY

The aim of the study was to study the immunogenicity of measles virus at a detailed level using different methods. As surface proteins of viruses are major targets of the immune system, the emphasis was put on analyzing the antigenicity and immunogenicity of surface proteins of MV, especially the hemagglutinin. The specific aims set for the study were:

1) to develop a method for measuring antibodies to MV surface components at a detailed level in different sera
2) to study the antibody response in different disease conditions;
3) to study the antigenicity of the MV hemagglutinin in humoral immunity using synthetic peptides;
4) to characterize the role of hemagglutinin in a proliferative assay using a mouse model.

3. MATERIALS AND METHODS

3.1 Cells and cell culture

Vero cells (African green monkey, kidney, ATCC CCL 81) were used for propagation of both wild type measles virus (MV) and vesicular stomatitis virus (VSV) and for plaque neutralization assay of MV. A laboratory MV strain isolated from an acute measles patient has been earlier described (Vainionpää et al., 1978). The VSV was of Indiana strain, a gift from Dr. L. Prevec , McMaster University, Hamilton, Ontario. For preparation of radiolabeled viral antigens, CV-1 cells (African green monkey, kidney, ATCC CCL 70) were used. Both Vero and CV-1 cell were routinely grown in Blake bottles with minimum essential medium (MEM, Auto-Pow medium, Flow Laboratories, Inglewood, California) supplemented with 5% fetal calf serum (FCS; Gibco, Laboratories, Grand Island, New York), 100 IU/ml penicillin G sodium, 0.1 mg/ml streptomycin sulfate, 0.002 M glutamine and 0.1% sodium bicarbonate.

3.2 Animals

Six to eight weeks old female Balb/c mice were obained from the breeding colony of the University of Alberta. SJL mice were purchased from Jackson Laboratories (Bar Harbor, ME). Flemish strain rabbits were ordered through Bioscience Animal Services of the University of Alberta.

3.2.1 Immunization of mice for antibody production

Inbred strains of Balb/c and SJL mice were used in this study. Ten mice of each strain were injected subcutaneously with 50 µg of purified wild type measles virus in Freund's complete adjuvant (FCA, Difco Laboratories, Detroit, MI). Blood samples were obtained by bleeding from the retro-orbital plexus under ether anesthesia seven days before

and at days 7, 14, 21 and 28 after virus injection. Plasma samples were diluted twenty-fold in EIA diluent (see below) and stored at -70°C until tested.

3.2.2 Production of antisera to the purified measles virions and synthetic peptides

Two Flemish strain rabbits were pre-bled and injectected intramuscularly in the gluteal and subscapular muscles with 0.5 mg of measles virions which were purified as described below and mixed with an equal volume of FCA. Three weeks later, 10 ml was bled from each animal for testing and each animal then immunized with 0.25 mg of the virions in incomplete Freund's adjuvant. This was repeated at three week intervals for three months and finally animals were boosted with 0.1 mg of virions in PBS intravenously and five days later they were anesthesized and blood was collected by cardiac puncture.

For raising of anti-peptide antisera, one Flemish strain rabbit per one peptide was pre-bled and injected intramuscularly in the gluteal and subscapular muscles with 0.5 mg of keyhole limpet hemocyanin (KLH) -conjugate of each synthetic peptide mixed with an equal volume of FCA. Three weeks later, animals were bled and boosted as described above. This was continued for three months after which animals were boosted once in every four weeks with 0.1 mg of the conjugate. After seven months of immunization, animals were boosted intravenously with 0.1mg peptide and five days later they were anesthesized and blood was collected by cardiac puncture.

3.3 Human sera

Sera included in this study were from 10 normal adult blood donors with a past uneventful measles infection in childhood, acute and early convalescent sera from 10 patients with uncomplicated measles infection (collected at the time of rash onset and seven to ten days after the onset of rash), and 10 SSPE patients. As a negative serum, a pool of sera taken from one to three year old unvaccinated children without a history of measles

was used. This pool did not bind in direct EIA to measles antigen. All the sera were obtained from the diagnostic unit of the Department of Virology, University of Turku, Finland.

3.4 Hemagglutination inhibition assay

HI assays were done as described by Norrby and Gollmar (1972). A previously determined amount of MV infected cell lysate antigen containing eight hemagglutinating units (HAU) in 25 µl was incubated with 25 µl of serially diluted serum samples. After one hour incubation at 37°C, 50 µl of washed monkey (*Cercopithecus aethiops*) red blood cells (1% packed volume) was added. After a further one hour incubation at 37°C, test results were read. In all steps, phosphate buffered saline (PBS) was used as diluting buffer.

3.5 Immunofluorescence

For IF tests, Vero cells were propagated and infected with MV as described above. When the infection had reached 75% cytopathogenic effect (CPE), the cells were washed with ice cold PBS and scraped off with a rubber policeman. After vigorous shaking, a single cell suspension was formed. IF with fixed and non-fixed cells was done. In the first instance, infected cells were centrifuged onto glass slides (Cytospin 2, Shandon, Astmoor, Cheshire, England). The cell were then fixed in ice cold acetone for 10 minutes. The rabbit antisera to be tested were diluted 1:100 with PBS and incubated on the cell spots in a moist chamber at 37°C for 30 minutes. Slides were then washed three to four times with PBS and incubated with an anti-rabbit fluorescein isothiocyanate (FITC) conjugate. After 30 minutes at 37°C, slides were washed three times with PBS and once with distilled water. Once the slides had dried, phosphate buffered glycerol was applied to the cell spots and covered with

plastic cover slips. Microscopic examination was done using a Leitz Dialux EB incident light microscope.

For surface fluorescence with non-fixed cells, the cells were prepared as described above. Approximately 1 x 10^6 cells were incubated with each antisera for 30 min in small plastic tubes in an ice bath. Cells were washed three times with PBS. This was followed by incubation with anti-rabbit FITC for 30 min in an ice bath and washing three times. The cells were applied to glass slides, dried, and examined.

3.6 Plaque neutralization assay

Vero cells were grown in 12 well culture plates (Nunc, Denmark) until confluent monolayers were formed. Inoculum virus (wild type MV) was diluted to give approximately 50 plaques per well and mixed with an equal volume of serial two-fold diluted serum samples in MEM + 2% FCS. The wells were emptied and serum-virus mixture was added to the cells and incubated for one h at 37°C in a humidified five per cent CO_2 atmosphere . Plates were shaken every 15 min to keep the monolayers covered with liquid. After one hour, an overlay of two per cent carboxymethylcellulose in MEM was added and incubated for six days. Plates were then stained and fixed with crystal violet in 20% ethanol and 10% formaldehyde for two hours, washed and the plaques counted.

3.7 Mouse T cell proliferation assays

The measles T cell proliferation assays were done as earlier described by Ziola et al. (1987). Five six to eight weeks old female Balb/c mice were injected intraperitoneally with 200 mg/kg of cyclophosphamide (CP; Horner, Montreal, Canada) dissolved in PBS. Two days afterwards, mice were immunized intramuscularly with eight µg of ß-propiolactone inactivated measles virus mixed with 100 µg of dimethyl dioctadecyl ammonium bromide

(DDA, Eastman Kodak). After seven days, draining popliteal, inguinal and axillary lymph nodes were removed from the mice and cells were separated using a fine steel mesh. Cells were washed three times with PBS and suspended in RPMI 1640 medium supplemented with eight per cent syngeneic mouse serum. The number of viable cells in the suspension was counted in a hemocytometer after dilution in 0.5% trypan blue. Stimulating antigens were diluted in PBS in a final volume of 50 µl and 2x 10^5 cells in 150 µl was added to each well of a microtiter culture plate. After two days incubation at 37°C in an atmosphere of five per cent CO_2, the proliferating cells were pulse-labeled for 16 h with 0.5 µCi of [^{3}H]-thymidine (Amersham Canada, Oakville, Ontario) and harvested onto glass fibre filters with an automated Titertek cell harvester (Eflab, Helsinki, Finland). The amount of incorporated thymidine was counted in Omnifluor (NEN Research Products, Boston, Ma.) scintillation fluid in a Beckman LS 9800 liquid scintillation spectrometer.

In peptide immunization experiments, several protocols were used. Mice were immunized with 0.1 mg of either free or conjugated peptides. As adjuvant, either FCA or DDA was used and the animals were immunized intracutaneously with the antigen-adjuvant mixture. After seven to nine days, draining lymph nodes were removed and single cell suspensions were made. In some experiments, animals were boosted after two weeks with the same antigen and the draining lymph nodes were removed after another week. This, however, was not found to improve the response and was not used in further experiments. In most experiments cells were cultured for four days before pulse-labeling with 1 µCi/well for six hours.

In certain peptide immunization experiments, spleens were taken from mice, cells were removed and layered onto Lympholyte M (Cedarlane Laboratories, Hornby, Ontario) density separation medium. The gradient was centrifuged for 20 min at 500 g and the lymphocyte layer was collected. Cells were washed three times, counted and passed through a column containing anti-mouse immunoglobulin coated glass beads (Sci-Can Diagnostics, Edmonton, Alberta). This column has been shown to be highly effective in

deletion of mouse B cells (Dr. D. Green, personal communication). Cells were then washed once, counted and cultured in U-bottom microwell culture plates as described above.

3.8 Delayed-type hypersensitivity assay

Delayed-type hypersensitivity assays (DTH) were done as described by Smith and Ziola (1986). The immunization protocol was identical to that used in the proliferation assay experiments but after seven days, mice were injected intracutaneously into the footpads with the challenge antigen. Footpad swelling was measured 24 h later. In the peptide DTH assay, CP was not used and mice were immunized with 25 μg of free peptide in DDA. Challenge was done with 20 μg of MV or the peptide.

3.9 Monoclonal antibodies to measles virus hemagglutinin and fusion proteins

The production and characterization of the MAb used in the competitive assay have been described previously (Sheshberadaran et al., 1983;Sheshberadaran and Norrby, 1986). These MAbs have been raised against LEC-KI strain of measles virus. By preliminary testing, four labelled MAbs were selected. These were I44 against H and 19HB4, 19GD6 and 9DB10 to F.

Seven MAbs were tested for their ability to bind to the synthetic peptides. These were I12, I29, I41, I44, 16CD11, 7AG11 and 16DE6, a gift from Dr. E. Norrby, Karolinska Institute, Stockholm, Sweden.

3.9.1 Purification and biotinylation of monoclonal antibodies

Immunoglobulin fractions were isolated from ascites fluids by precipitation with 18% sodium sulfate. Salt was removed from the precipitates by dialysis against the chromatography buffer used in the procedure. The specific antibody fraction was isolated with a Mono-Q anion exchange column (Pharmacia, Dorval, Quebec) connected to a high performance liquid chromatography system operating at a back pressure of 1200 kPa (Varian 5060 LC and Vista 401 controller, Varian Instruments , Walnut Creek ,CA). The buffers used in Mono Q were 20 mM Tris, pH 7.7 (buffer A) and same with 0.35 M NaCl (buffer B). The column was equilibrated with 50 ml of buffer A and sample loaded into the column in the same buffer. After washing the non-bound material out with 5 ml of buffer A, a linear gradient of buffer B was run so that the concentration of B increased from 0 to 100% in 16 ml volume. Fractions (0.5 ml) were collected and the OD_{280} measured. Fractions corresponding to the peaks were tested in direct enzyme immunoassay on cell lysate antigen prepared from measles virus infected cells (see below). The antibody containing fractions were pooled and dialyzed against several changes of PBS, pH 7.4. Immunoglobulin concentrations were determined by measuring the OD at the wave length of 280 nm and dividing this by the extincition coefficient of 1.4 for mouse immunoglobulins. The immunoglobulin concentration was adjusted to 1 mg/ml. N-hydroxysuccinimidobiotin (Sigma, St. Louis, Mo.) was dissolved in 25% N', N'-dimethylformamide (Fisher, and added to immunoglobulin solutions at a molar ratio of 100:1 (biotin:immunoglobulin). The mixture was incubated at room temperature for three hours and dialyzed extensively against PBS. The aliquots were stored at -70°C until used.

3.10 Viral antigens

3.10.1 Purified viruses

Vero cells grown in roller bottles were infected with the wild type MV at a multiplicity of infection (m.o.i.) of 0.01. When CPE reached 75 to 100%, the medium

was harvested and cell debris removed by centrifugation at 500 g for 15 min. The medium was concentrated with a Millipore Minitan concentration apparatus (Millipore, Mississauga, Ontario) and centrifuged for two hours at 80,000 g (Spinco SW 27 rotor) in a 20 and 60% (w/w) sucrose step gradient at 4°C. The material at the interface was collected and centrifuged in a sodium potassium tartrate step gradient (18%, 30%, 40%, 50%) for three hours at 80,000 g(Spinco SW 27 rotor). The collected virus material was diluted in GNTE buffer (0.2 M glycine, 0.2 M sodium chloride, 0.02 M Tris-HCl, 0.002 M EDTA, pH 7.8), pelleted at 80,000 g for one hour and resuspended in GNTE.

VSV was propagated in Vero cells and when CPE reached 75-100%, the medium was harvested, cell debris removed and the supernatant concentrated as described for MV. The concentrated material was layered on a 20% sucrose cushion. Sucrose was diluted in TNE buffer (0.05 M Tris, 0.001 M EDTA, 0.15 M NaCl, pH 7.4). Virus material was centrifuged at 80,000 g for two hours through the sucrose and the pellet was suspended in TNE and sonicated. This material was then layered on a linear 20-60% sicrese gradient and centrifuged at 80,000 g for a minimum of four hours. The virus band from the gradient was collected and pelleted by centrifugation at 80,000 g for two hours.

3.10.2 Cell lysate antigen

MV infected cells were harvested when CPE was complete. The cells were washed and resuspended in PBS and homogenized in a Sorvall R Omnimixer (Norwalk, Conn.). The material was centrifuged at 600 g and the supernatant centrifuged for 30 min. at 80,000 g. The pellet was suspended in PBS. The antigens were stored at -70°C and sonicated before use as antigen in EIAs.

3.10.3 Endoglycosidase treatment of the purified virus

Purified wild type MV preparation (0.3 mg total protein) was dialyzed against 0.1 M Na_2HPO_4, 0.05 M citric acid, pH 5.0. Half of the preparation was incubated with 10 mU of endoglycosidase H at 37°C overnight and the other half of the preparation was incubated the same way but without the enzyme. Both the control and the enzyme treated

virus were coated at a concentration of 3 µg/ml on microtiter EIA plates for testing of the antisera.

3.10.4 Preparation of MV/Vero membranes and lentil lectin isolation of hemagglutinin

Vero cells were infected with wild type MV as earlier described. Infected monolayers at 75 to 100% CPE were washed two times with cold PBS and then scraped into PBS followed by pelleting at 400 g for 10 min at 4°C.

The cell pellet was resuspended in 5 ml of 1:10 PBS and emulsified in a Dounce homogenizer (10-15 strokes) on ice. Nuclei were pelleted at 400 g for 10 min at 4°C and 5 M NaCl was added to the supernatant to give a final concentration of 0.1 M. Membranes in the supernatant were pelleted by centrifugation at 90,000 g (SW 60 rotor) for 30 min at 4°C. The membranes were then resuspended in 1-2 ml of PBS. Hemagglutination titration of different preparations gave H titres of 2^6 to 2^{10}. Ten per cent NP-40 was added to the membranes to a final concentration of 2.0% and incubated on ice for 10-15 min. An equal volume of 0.01 M $NaHPO_4$, pH 7.1 + 1.0% deoxycholate (DOC) was added, mixed at room temperature, centrifuged for one minute at 10,000 g, and the supernatant removed. The supernatant was placed onto a 2 ml bed volume column of lentil lectin (LL) Sepharose (Sigma, St. Louis, MO), equilibrated in 0.01 M $NaHPO_4$, pH 7.1 + 1.0% DOC. The column was then washed with 25-30 bed volumes of the same buffer and the glycoproteins eluted with 0.1 M alpha-methyl-D-mannoside in column buffer. The OD_{280} of the column fractions was read and 1 µl aliquots of the fractions were added to EIA plate wells containing 100 µl of PBS. Wells were coated overnight and tested with rabbit anti-MV H serum (1:500 dilution). The OD_{280} and EIA peaks indicating H coincided.

The H protein peak was pooled and NP-40 added to a concentration of 0.1%. The pool was then dialyzed against PBS + 0.1% NP-40, concentrated using Sephadex G-200 and the pool dialyzed extensively against PBS + 0.1% NP-40.

3.10.5 Preparation of MV nucleocapsid from MV infected Vero cells

Preparation of NCs was done according to Lund et al. (1984). Vero cells were infected as earlier described and when CPE reached 90-95%, cells were washed twice with cold PBS, scraped from the surface and pelleted. Cells were suspended in PBS containing 1% Triton X-100 and kept on ice for 30 min. The nuclei were removed by centrifugation (1000g, 20 min), the supernatant was collected and layered onto a step gradient of 25% (w/v, 6 ml), 30% (4 ml) and 40% (2 ml) CsCl in PBS. The gradient was centrifuged at 4°C at 85,000 g (SW27 rotor) for 90 min. The NC band was collected, diluted 1:1 with PBS and layered onto another step gradient (4 ml of 20, 25, 30, 35 and 40% CsCl in PBS). The gradient was centrifuged in a SW27 for 16 h at 80,000 g, the NC band harvested and pelleted at 100,000 g, 30 min. The pellet was resuspended in PBS.

3.10.6 Isolation of MV hemagglutinin from SDS-PAGE

Purified MV was disrupted and separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as follows. Following electrophoresis, the gel was rinsed briefly with PBS containing 4 M urea. A marker lane containing H protein was removed and stained with Coomassie blue. This was lined up with the rest of the gel and the H region cut from the gel. The acrylamide gel was passed through an 18 g needle and the gel resuspended in PBS containing 4 M urea (2-3 ml). The gel was extracted overnight at 37°C (end-over-end mixing) and for one to two days at 4°C. The gel was then filtered from the solution and the protein solution dialyzed for three days at 4°C against three changes of 2 l of PBS. Protein concentration of this antigen was approximately 5 µg/ml. When coated in EIA plate wells, the antigen reacted only with anti-H monoclonal antibody and not with P, NC, M or F monoclonals.

3.10.7 Affinity chromatography purification of measles virus hemagglutinin

MV H protein was isolated by LL column chromatography as described above and dialyzed against RIPA buffer (0.01 M Tris-HCl, pH 8.2 + 0.1 M NaCl + 0.6 M KCl + 0.5

mM MgCl2 + 2.0% Triton X-100). Dialyzed H was placed onto a column of Affi-Gel 10 (Bio-Rad, Mississauga, Ontario) to which a mixture of four anti-H MAbs (16CD11, I29, I41 and I12 from Dr. E. Norrby, described by Sheshberadaran, 1985) was coupled. Sodium sulfate immunoglobulin fractions of the MAb in PBS were coupled to the Affi-Gel as per supplier instructions. The column was equilibrated in RIPA buffer. Following loading, the column was washed with 10 to 20 column volumes of RIPA buffer, 10 to 20 column volumes of RIPA buffer + 0.05% SDS and 3 to 4 volumes of 0.1 M $NaHPO_4$, pH 7.1, + 0.1% NP-40. The H protein was then eluted in the same buffer containing 3.5 M KSCN. Column fractions were monitored by EIA using 1 µl aliquots. The H peak was pooled and dialyzed against PBS + 0.1% NP-40.

3.10.8 Preparation of MV hemagglutinin liposomes

The dialyzed pool from the Affi-Gel column was loaded onto a 1.5 ml column of DEAE-Sepharose equilibrated in 0.01 M $NaHPO_4$, pH 7.1 + 0.1% NP-40. The column was washed with four to five volumes of 0.01 M $NaHPO_4$ pH 7.1 + 0.1% NP-40, followed by three to four volumes 0.01 M $NaHPO_4$, pH 7.1 + 30 mM octyl glucoside and the H eluted by inclusion of 1.0M NaCl in the octyl glucoside buffer. Fractions were monitored by EIA as per the previously described technique. The H peak was then pooled.

For liposome preparation, 2 x 2 µl aliquots of phosphatidylcholine (Sigma, St. Louis, MO) in hexane (200 µg each) were blown dry in two glass test tubes (using an air line), resuspended in five drops of ether and blown dry again. To one tube, 1.9 ml of the H pool eluted from the DEAE column was added. To the other was added 1.9 ml of 0.01 M $NaHPO_4$, pH 7.1 + 1 M NaCl + 30 mM octyl glucoside (for control liposomes). Both preparations were dialyzed at 4°C for four days against four changes of 2 l of PBS.

3.10.9 Preparation of VSV/Vero glycoprotein antigens

VSV and Vero membranes, glycoprotein fractions and liposomes were prepared in the same manner as MV material.

3.10.10 Virus inactivation

The MV and VSV were inactivated with ß-propiolactone (Sigma, St. Louis, MO) which was diluted in freshly made 0.2 M $NaHCO_3$ containing 0.14 M NaCl. This solution was mixed with each antigen to a final concentration of 0.1% (v/v). The mixtures were shaken at 4°C for two hours and transferred to 37°C for overnight incubation. After this, the antigens were aliquoted and stored at -20°C until used.

3.11 Enzyme immunoassays

The solid-phase immunoassay method employing microtiter plates was used throughout. Preliminary studies showed that purified virus was needed for optimal results with anti-F MAbs whereas cell lysate antigen was equal to purified virions and was used when antibodies to the H-polypeptide were tested. Microtiter plates (Linbro, Flow laboratories, Mississauga, Ontario) were coated with either 300 ng/well (0.1 ml/well) of purified virions or 700 ng/well of cell lysate antigen. After overnight incubation at room temperature, free binding sites on the plastic plates were blocked with 0.2 ml/well of EIA diluent (PBS supplemented with 0.5% bovine serum albumin (BSA), 0.5% Tween 20 and 0.1 mM merthiolate). After an incubation period of 60 to 90 min. at 37°C the plates were used in the assays.

For competition binding assays, serial four-fold dilutions (starting at 1:200) of the sera were incubated in antigen-coated plates overnight at room temperature. The plates were washed three times with a washing solution (PBS supplemented with 0.1% Tween 20) and a previously determined optimal amount of biotinylated MAbs giving OD_{280} values of about 1.0 were added. After a 60 min. incubation at 37°C the plates were washed as above and horseradish peroxidase (HRPO)-labelled streptavidin (Amersham, Oakville, Ontario) was added. After a further 60 min. incubation at 37°C the plates were washed four times and 0.1 ml/well of substrate solution (30 mg *o*-phenylenediamine plus 7 µl of

30% hydrogen peroxide in 10 ml of 0.1 M citrate buffer, pH 5.5) was added. After 15 to 30 min. incubation in the dark the reaction was terminated by adding 0.1 ml/well of 1N HC1 and optical densities (OD_{492}) were read with a Titertek Multiskan photometer (Eflab, Helsinki, Finland).

In competition binding assays, binding of negative serum to viral antigen was taken to represent background and binding of each labelled MAb to antigen as maximum binding. The difference was taken to represent 100% binding and relative inhibition by the competing sera was calculated on this scale. The level for defining titers of the antibodies was set to 75% of maximal binding as even high concentrations of non-specific antibodies could not cause more than 20% inhibition.

In direct binding assays, microtiter plates were coated with antigen as described above. When the synthetic peptides were used as a solid-phase antigen, wells were coated with either 10 μg/ml of BSA-conjugated peptides or 15 μg/ml of non-conjugated peptides and the MV antigens the same way as described above. After blocking the non-specific binding with the diluent buffer, the sera to be tested were incubated on the wells for one hour at 37°C. After washing the plates, the HRPO-conjugated species specific anti-immunoglobulin (anti-mouse, -rabbit or -human) was incubated at a pre-determined optimal dilution on the wells and the rest of the assay was done as described for the competitive assays.

3.12 Synthetic peptides

The sequence of the Edmonston strain of MV H (Alkhatib et al., 1986) was used for selection of peptides to be synthesized. The analysis for predicting surface exposed areas of H protein was done using the Surfaceplot program (Synthetic Peptides Incorporated, University of Alberta) with an IBM PC computer. The selected peptides were puchased from the Alberta Peptide Institute (Edmonton, Alberta). Ten to eleven amino

acid long peptides were synthesized and conjugated to the carrier as described by Parker and Hodges (1985). As carriers, KLH and BSA were used. Two of the peptides, H-6 and H-9, were also received in free form, i.e. not conjugated.

3.13 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide (SDS-PAGE) gel was prepared using a modification of the method described by Laemmli (1970). The 10% slab-gel was done as follows. Ten percent acrylamide was diluted from 30% stock solution into 0.4 M Tris, pH 8.8, containing 0.1% SDS and 0.05% $(NH_4)_2SO_4$. Prior to pipetting the solution into the gel apparatus (Bio-Rad Protean II, Bio-Rad, Mississauga, Ontario), 15 µl of tetramethylethylenediamine per 60 ml acrylamide solution was added. After polymerization of the resolving gel, stacking gel was added. Three per cent stacking gel in 0.12 M Tris, pH 6.8, was used and the other ingredients were same as in the resolving gel. The samples were diluted in 0.1 M Tris pH 6.8 containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol and boiled for two minutes in water bath. The gel was electophoresed overnight at 6 mA.

For silver staining, the gel was fixed in acetic acid:methanol:water (1:5:4) for four hours and washed five times with H_2O. Staining was done with a solution containing 0.02 N NaOH, 0.1 N NH_4OH, and 0.6% $AgNO_3$. After 20 minutes, the gel was washed five times with H_2O and color developed by adding 2.5 ml of 0.1% citric acid and 0.25 ml formaldehyde in 500 ml H_2O. The reaction was stopped with 7% acetic acid. In certain cases, Coomassie Brilliant blue was used for staining of the gel. The gel was stained for five minutes with the dye diluted in acetic acid:methanol: water (7:5:88) to give a final concentration of 0.2%. Destaining was done with the same solution without the dye.

3.14 Western blotting

Purified MV was separated on SDS-PAGE as described above. For electrophoretic transfer of proteins, a sandwich was made containing from the top, two layers of Whatman no. 1 filter paper, nitrocellulose paper (to the negative side), the gel and two layers of filter paper. Transfer was done in a chamber filled with buffer containing 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3 at 30 V for threee hours. To localize polypeptides, heparin-toluidine staining was used. The nitrocellulose was soaked in 6 M urea for three minutes, washed six times with H_2O, treated with heparin (200 U/100 ml of 10 mM HCl, pH 2) for 10 min, washed five times with H_2O and stained with 0.02% toluidine blue in 10 mM HCl. The stained filter was cut into strips which were destained with 8% acetic acid, 50% methanol, 42% H_2O. Destained strips were washed with TBS (10 mM Tris-HCl, pH 7.4 + 0.9% NaCl). For immunological staining, strips were soaked in TBSA (3% BSA in TBS) for three hours at 37°C and rinsing in the same solution. Sera were diluted 1:50 in TBSA and reacted with blots for one hour at 37°C, washed four times with TBS, followed by one hour incubation with anti-rabbit peroxidase (Biosys, France) at 37°C. As a substrate, 1% *O*-dianisine dissolved in methanol was freshly diluted with TBS to a final concentration of 0.01% and 0.3% H_2O_2 was added. The reaction was stopped after five minutes with H_2O, and the strips were washed and air dried.

3.15 Radio-immunoprecipitation

Radiolabelled virus antigen was prepared as follows. Confluent monolayers of CV-1 cells were infected with MV at an approximate m.o.i. of 1. After CPE had reached 30-40%, monolayers were washed with sterile PBS and methionine-free medium supplemented with [^{35}S]-methionine (50 µCi/ml) was added. When CPE had reached 100%, cells were scraped from the surface and washed with cold PBS. Cells were then

lysed with RIPA buffer (2% Triton X-100, 0.1 M NaCl, 0.6 M KCl, 0.5 mM $MgCl_2$, 1 mM phenylmethylsulphonyl-fluoride and 1% aprotinin in 0.01 M Tris-HCl, pH 8.2) on ice for 30 min and centrifuged at 80,000 g for one hour. The supernatant was collected and stored at -20°C.

For immunoprecipitation, 20 µl of serum samples were added to 200 µl of labelled lysate and the total volume adjusted to 500 µl with RIPA buffer. The immunoreaction was done on ice for three hours after which antigen-antibody complexes were precipitated using 100 µl of protein A-Sepharose CL-4B (Sigma, St. Louis, MO) in PBS. The mixture was kept on ice for one hour and thoroughly mixed several times after which the beads were removed by centrifugation. Pellets were washed five times with RIPA buffer.

Precipitated complexes were then electrophoresed in SDS-PAGE as described above. The gel was fixed in 7% acetic acid + water/methanol (7:3) for several hours, soaked in Amplify (Amersham, Oakville, Ontario) for 30 min and dried under vacuum at 60-80°C. The dried gel was then held in close contact with Kodak X-Omat AR-50 film for seven days at -70°C and the film was developed.

3.16 Protein determinations

All protein determinations were done using the Bio-Rad protein assay (Bio-Rad Laboratories Canada, Mississauga, Ontario) with BSA as a standard.

3.17 Statistical methods

The significance of the differences between antibody titers in different patient groups was analyzed using non-parametric Mann-Whitney U test in the Statview program in a Macintosh computer. The same test was also used for determining the significance of differences between the stimulating antigens in the T cell assays.

4. RESULTS

4.1 Purification of monoclonal antibodies

For competitive immunoassays, immunoglobulins were purified from ascites fluid by a two-step technique. After the first step of sodium sulfate precipitation, precipitates were dialyzed extensively against 20 mM Tris buffer, pH 7.7, and the samples were filtered through a 0.22 μm filter before loading them onto a Mono Q anion exchange column. Considerable variation was found in the OD_{280} elution profiles obtained with different samples. As an example, the profile of MAb 19HB4 is presented in Figure 1. In most cases, the immunoglobulin fraction eluted at 0.25-0.3 M NaCl. The optical density at 280 nm of each fraction was measured and tested for antibody activity by EIA. The major activity was found in peak 2 of the Mab 19HB4 elution profile. Samples of two purified MAbs were also tested on SDS-PAGE for purity. Both samples contained only heavy and light chains immunoglobulins when the gel was stained with Coomassie brilliant blue (data not shown).

Ascites fluids sometimes contain considerable amounts of other proteins including transferrin and albumin (Clezardin et al., 1985). Based on these results, each ascites fluid seems to be unique in the amount of contaminating proteins (precipitation conditions being the same). In some cases, the precipitate contained only one major peak in anion exchange chromatography.

4.2 Competition assays for polypeptide and antigenic site specificity of monoclonal antibodies to hemagglutinin and fusion proteins

The polypeptide and antigenic site specificity of the MAbs was characterized using a competition EIA as shown in Figure 2. When MAb I44 (anti-H) was used as a competing

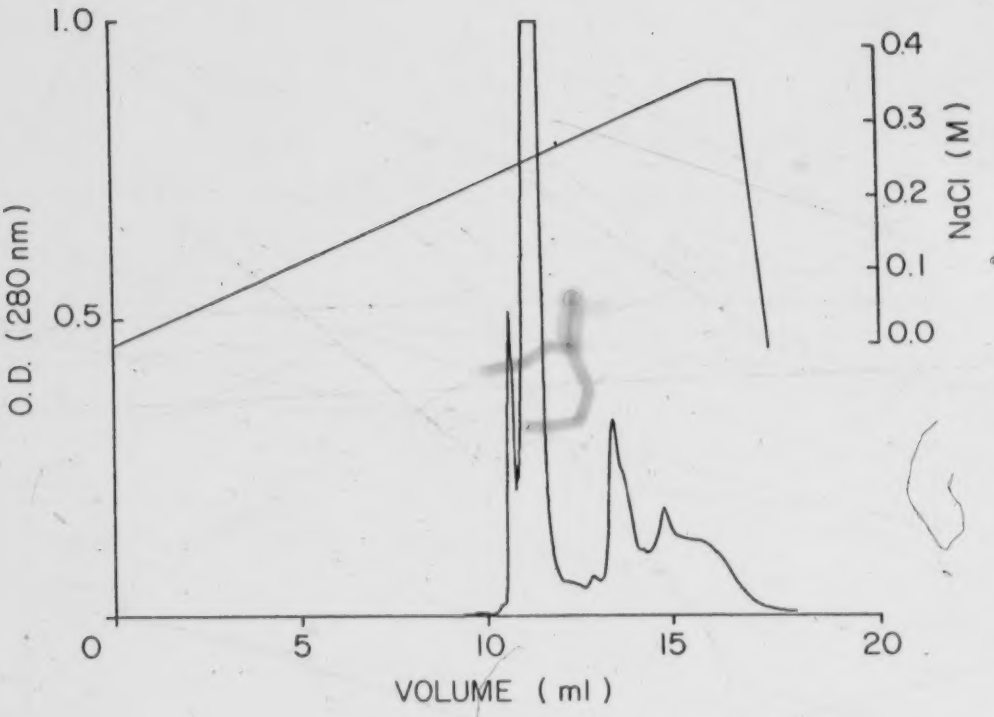

Figure 1. High performance liquid chromatography profile of purification of MAb 19HB4. The sample was applied to a Mono Q anion exchange column in 20 mM Tris, pH 7.7. Elution was done with an increasing salt gradient in the same buffer.

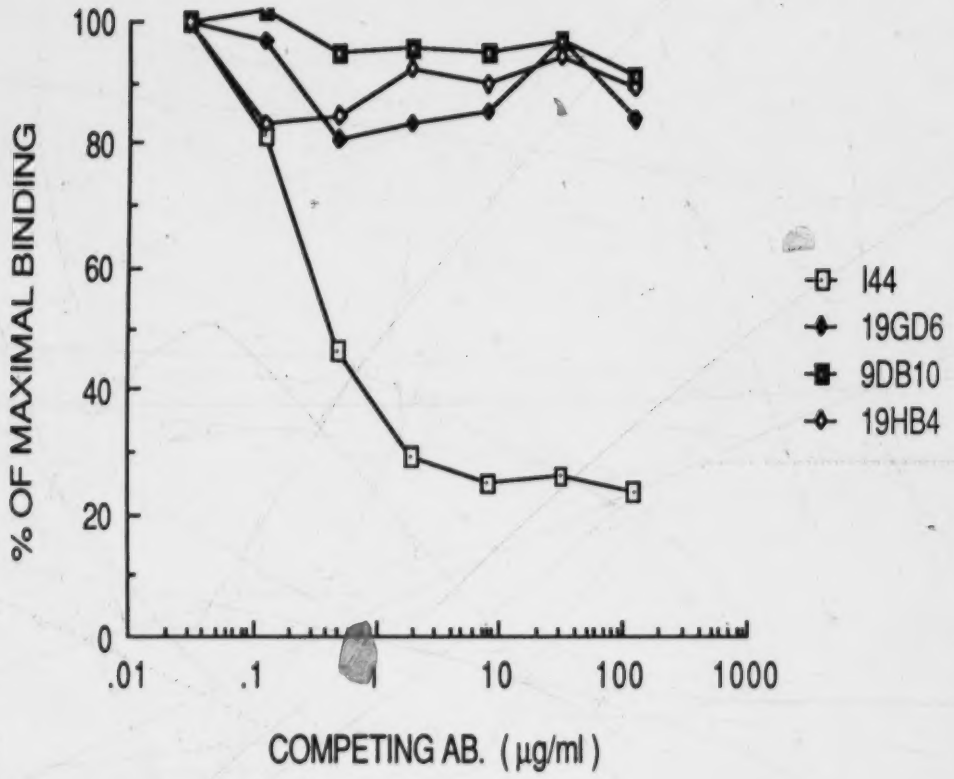

Figure 2. Homologous and heterologous inhibition of the binding of MAbs to MV antigen. Unlabelled MAb against MV H (MAb I44) was incubated on the plates followed by incubation with biotin-labelled MAb I44 or 3 MAbs against F protein, 19GD6, 9DB10 or 19HB4.

unlabelled antibody, it blocked the binding of labelled MAb I44 but did not block the binding of the F protein-specific MAbs 19GD6, 19HB4 or 9DB10 . Similar experiments were done with all the labelled antibodies, the results of which were comparable to those presented in Figure 2. These experiments demonstrated that antibodies to MV H and F proteins could be measured separately under these experimental conditions.

When three MAbs against the F protein were used in the competition binding assay some overlapping in binding of antibodies to different epitopes was found. However, two major antigenic sites could be distinguished on the F protein. One site was defined by the MAb 19GD6 or 19HB4 and another site was recognized by MAb 9DB10 when 19GD6 was used as the unlabelled antibody (Figure 3). Although low level cross-inhibition overlapping was seen between these sites, there was an almost hundred-fold difference between the MAbs in antibody concentrations causing 25% inhibition. This was also confirmed using MAb 19HB4 as a competing antibody, which did not block the binding of MAb 9DB10 (data not shown). The results show that antibody responses against these two distinct antigenic sites on the F-polypeptide can be measured with this technique.

4.3 Antibodies specific to polypeptides and antigenic sites in human sera

Antibody titers against the H polypeptide and against two different antigenic sites on the F protein were measured using competitive inhibition assays. Results of a typical assay are presented in Figure 4. The antibody titers were set at the level corresponding to 25 % inhibition of the maximal binding. Antibodies to the antigenic sites recognized by the four mouse MAbs were present in each serum group.

All the antibody titers measured increased significantly from acute to early convalescent sera from measles patients. The lowest mean titer in acute sera was against the site on the H protein; only one of the patients had a higher log 2 titer than 7.6 (threshold level). The highest mean titer in both acute and early convalescent sera was against the

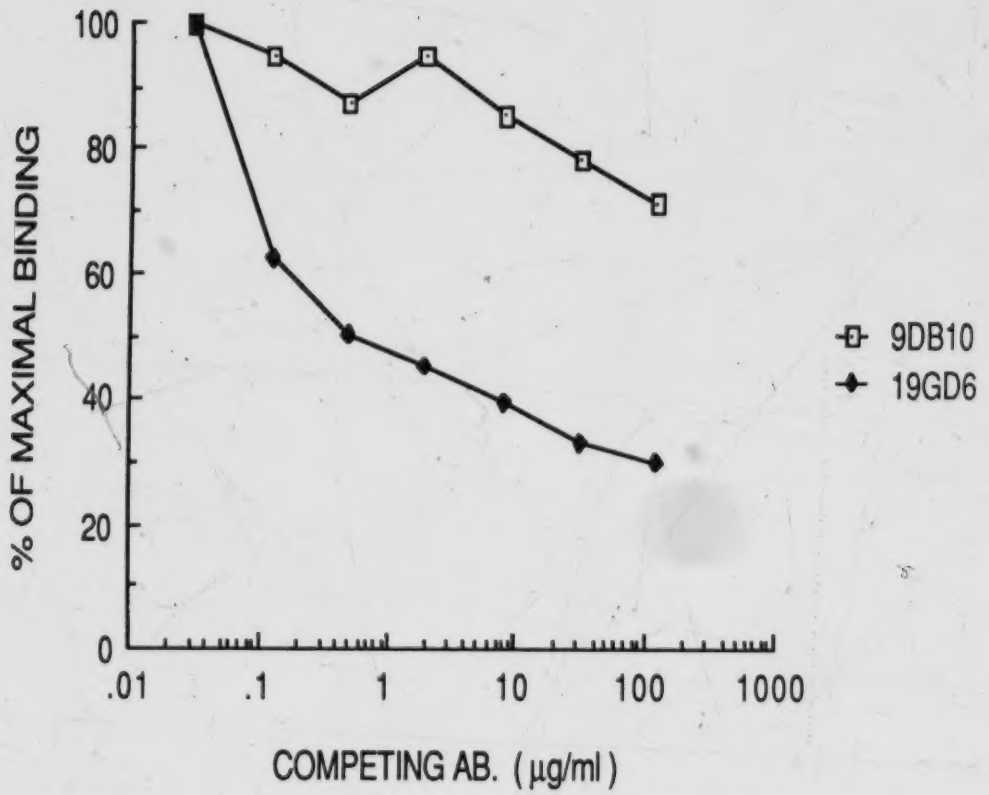

Figure 3. Inhibition of binding of 2 different MAbs to MV antigen. Unlabelled MAb 19GD6 inhibits the binding of the labelled homologous antibody but does not significantly inhibit the binding of another MAb (9DB10) to the same polypeptide.

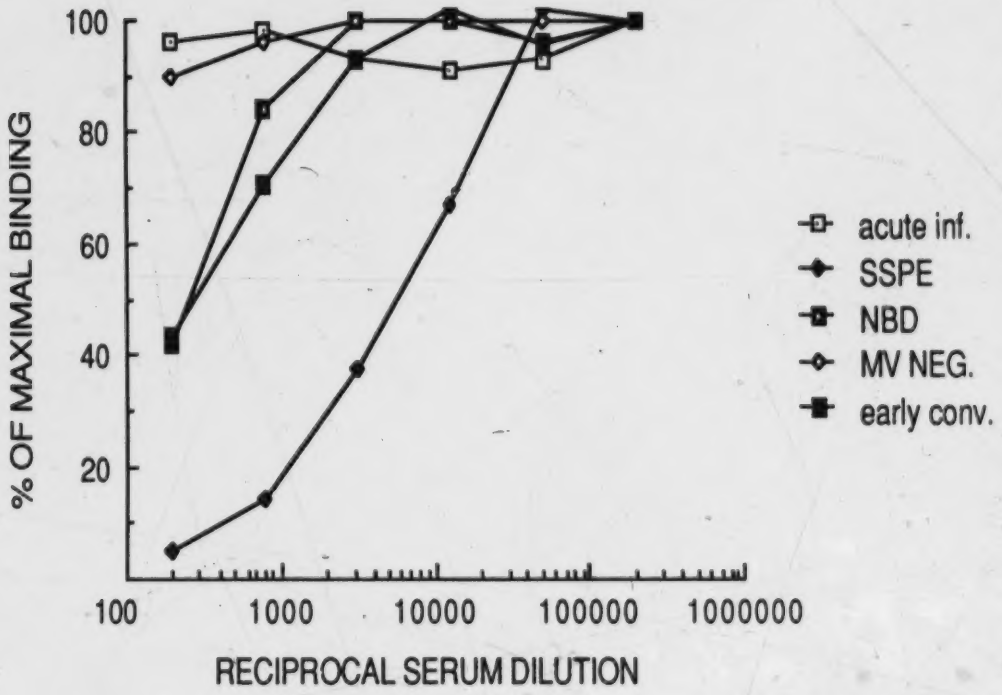

Figure 4. A typical inhibitory assay for measurements of antibodies in human sera. Different human sera were incubated on antigen plates and the binding inhibition of labelled MAb I44 was measured. One measles-negative serum, 1 serum taken from the acute and early convalescent phases of a measles patient, 1 serum from a NBD with past measles history and 1 SSPE were used in this test.

antigenic site on the F protein defined by MAb 19HB4. As well, the titer increase was largest for these antibodies (see Table 1).

Table I. Mean titers and standard deviations (log 2) of antibodies to different antigenic sites on H and F proteins. Ten patients each from NBDs, patients with acute measles or convalescing from it, and SSPE patients were tested.

Antigenic site defined by MAb	NBD	Acute	Convalescent	SSPE
I44	8.9±1.4	<7.1	9.9±0.7	13.4±1.6
19GD6	8.0±1.0	8.7±1.4	12.1±0.6	13.5±1.4
9DB10	8.2±0.8	7.8±0.5	10.1±1.2	11.6±1.1
19HB4	8.3±0.7	9.0±1.4	13.0±0.6	13.2±1.4

The antibody titers to the F protein were lower in NBDs than in early convalescent sera (Mann-Whitney U test, p values ranged from 0.05 to 0.01). Although the titer against the epitope on the H protein was low in relation to titers against the F protein in early convalescent sera, this was the highest mean titer in the NBD group. This would indicate that the antibody response to this particular site is developed late during the infection. In SSPE patients all titers were considerably higher, in accord with the known hyperimmune state of these patients (Salmi et al., 1972). Only one of the mean titers (see Table 1), defined by MAb 9DB10, was clearly lower than the others (p values ranged from 0.05 to 0.02).

The relative amounts of antibodies against the antigenic sites was assessed by calculating antibody titer ratios. A significant difference between the NBD and SSPE patients ($p<0.02$, Mann-Whitney U test) was seen in MAb 19HB4/9DB10 antibody titer

(anti-F) ratios which were higher in SSPE patients (see Figure 5). All anti-F/anti-H ratios were found to be close to each other in both SSPE and NBD groups whereas early convalescent sera had significantly higher ($p<0.01$) ratios.

4.4 Antibody response in two mice strains after immunization

All antibodies defined by the four MAbs were present in sera of both Balb/c and SJL mice after immunization with purified measles virions. All antibody titers increased until day 28 in both strains (Figure 6). The antibody titers to F protein were significantly higher on day 28 p.i. in Balb/c than in SJL mice which reflects the fact that total antibody titers were also significantly higher in Balb/c mice (data not shown). However, the anti-H antibody titers did not differ significantly between the two strains. Anti-F/anti-H antibody titer ratios were thus higher in Balb/c mice than in SJL mice. A difference between these mouse strains was also found in the antibody titer ratios of the F protein epitopes 19HB4/9DB10. SJL mice had higher ratios on days 7 to 28 (see Figure 7) than did the Balb/c strain.

4.5 Synthetic peptides

4.5.1 Selection of regions of hemagglutinin for synthesis of peptides

A computer program combining hydrophilicity, accessibility and flexibility parameters of amino acids was used to predict primary sequences of the MV H protein likely to be exposed at the surface of the molecule. Alkhatib et al. (1986) sequenced the cDNA corresponding to the H mRNA from the Edmonston strain of MV and the amino acid sequence derived from this was used in the prediction. The hydrophilicity and composite profiles of the H protein are shown in Figure 8. Based on the composite graph, ten sites were selected which were predicted to be exposed on the surface of the molecule.

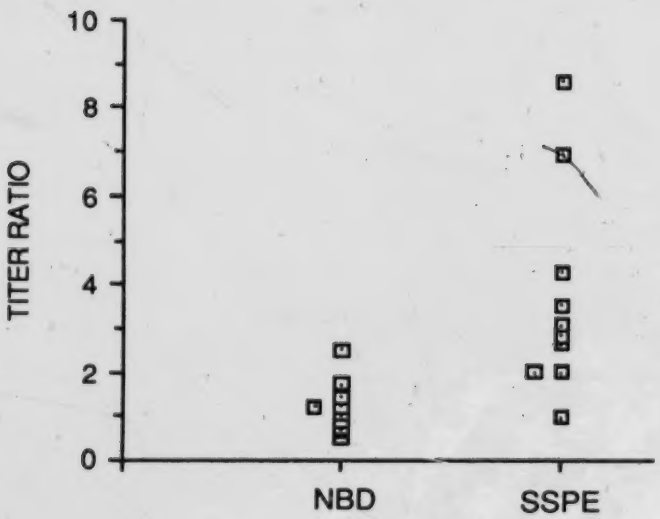

Figure 5. Ratio of anti-F antibody titers (MAb 19HB4/MAb 9DB10) in human sera. Serum specimens from 10 NBDs and 10 SSPE patients were tested.

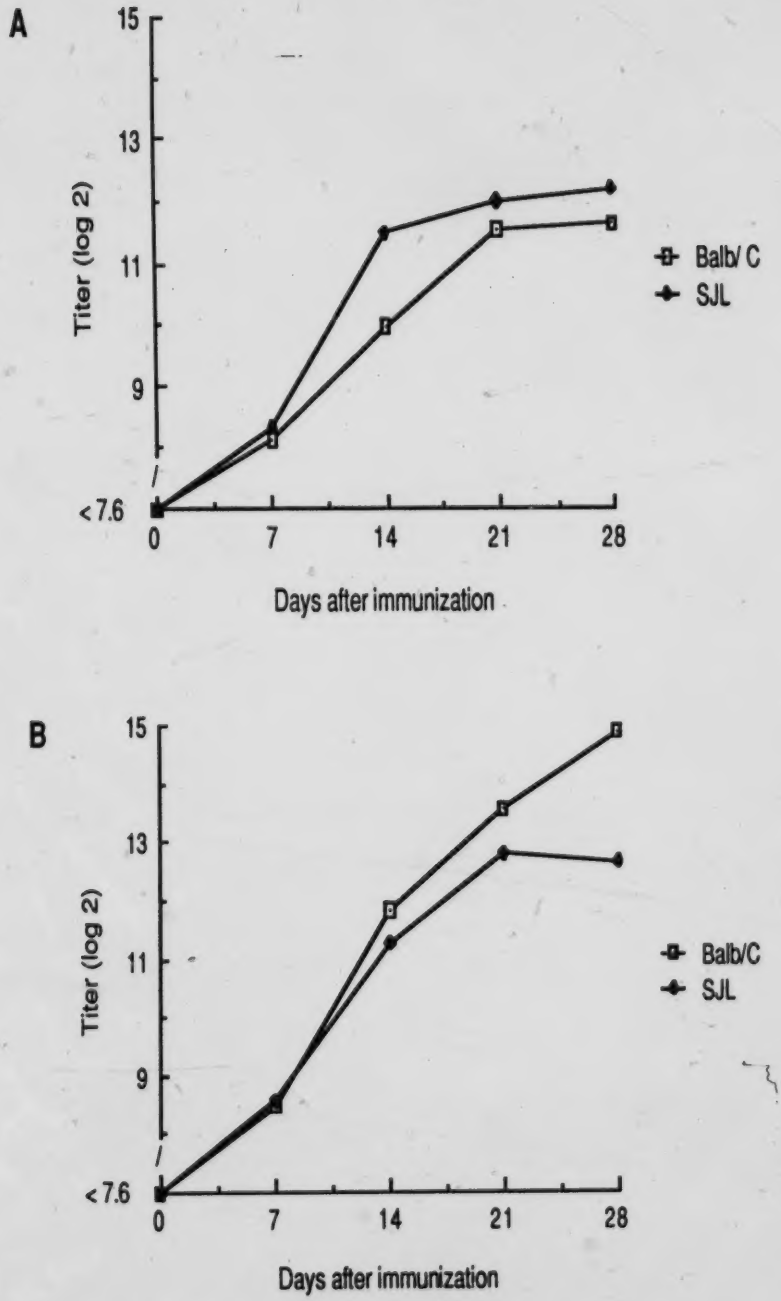

Figure 6. Kinetics of antibody synthesis against the H epitope MAb I44 (A) and the F protein epitope MAb 19HB4 (B) in 2 inbred mouse strains.

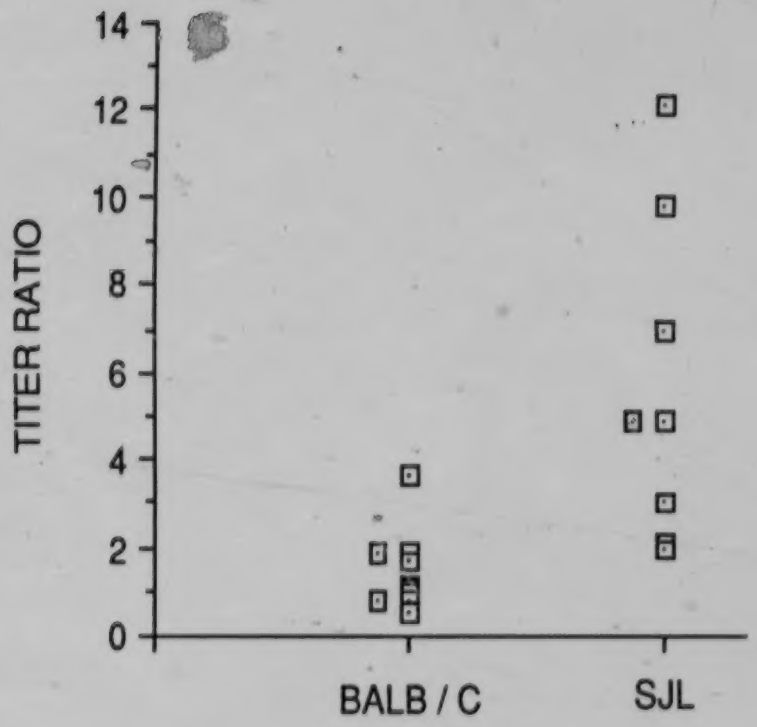

Figure 7. Ratio of antibody titers (MAb 19/HB4/MAb 9DB10) in Balb/c and SJL mice at 28 d after immunization.

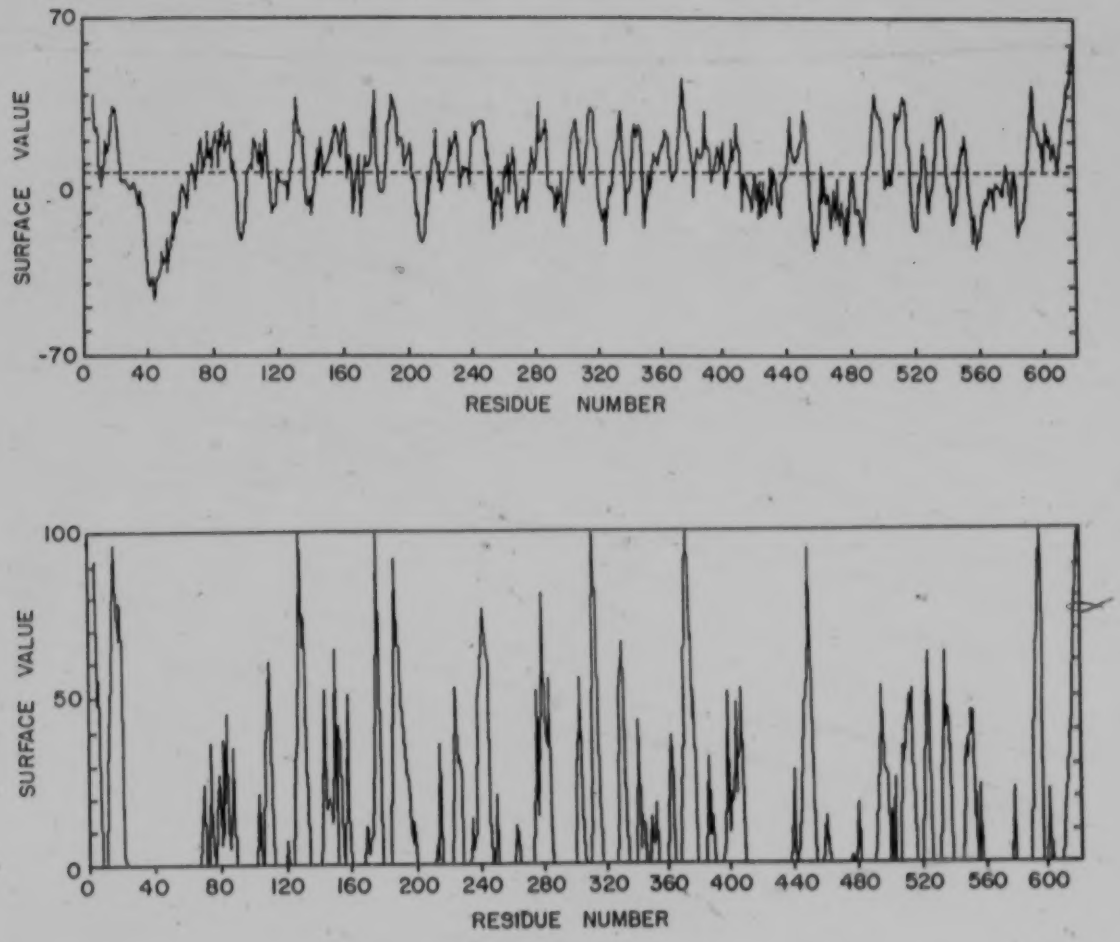

Figure 8. Surface profiles of MV H. Hydrophilicity profile (A) derived from parameters by Parker et al. (1986) and composite profile (B) combining hydrophilicity, accessibility and flexibility parameters of amino acids are presented. The profiles have been obtained by summing the parameterers for each amino acid of a seven-residue segment and giving this value for the fourth residue. This procedure is then repeated for the whole molecule as a 'gliding window' from the amino- to the carboxyterminus.

Four 10-11 amino acid -long peptides were synthesized corresponding to regions mainly of the middle part of the H protein.

The Hallé strain sequence (Gerald et al., 1986) was also analyzed but, as expected from the sequence homology, the profiles were virtually identical to the Edmonston strain (data not shown). Another sequence derived from the Edmonston strain (W. Bellini, unpublished) was compared to that of Alkhatib and again, no major differences were found. In the H-5 peptide (see below), however, Alkhatib reported a cysteine residue in position 188 whereas Bellini detected tyrosine. However, the Hallé strain also contained a cysteine at this site. Four peptides were selected from the middle portion of the protein for synthesis and the sequences of these are given in Table 2. Prediction of the contribution of these peptides to the secondary structure was also done using a computer program (Microgenie, Beckman Instruments, Mississauga, Ontario). This program predicts ß sheets, α helixes, random coils and turns for the protein. The analysis is done for the whole protein and the program gives for each amino acid a symbol indicating which structure the residue is predicted to be part of in the native protein. This information is provided in the Table 2.

4.6 Reactivity of hyperimmune sera, monoclonal antibodies and human sera with synthetic peptides

Several different sera were tested for their ability to bind to BSA-conjugated synthetic peptides in a direct EIA. Three of the peptides, H-6, H-9 and H-10, were recognized by polyclonal rabbit serum against whole virus as shown in Figure 9. Binding to H-9 was best in both titer and affinity as suggested by the binding curve. Another hyperimmune serum tested gave similar results. In all cases, however, the reactivity was at relatively low level with the highest titer slightly above 1:1000.

Table 2. Amino acid sequences of the synthesized peptides.*

Peptide	Residue numbers	Sequence
H-5	185-195	LysGlyAsnCysSerGlyProThrThrIleArg T T T T R R T B B B B
H-6	235-245	GluLysProAsnLeuSerSerLysArgSerGlu B B T T A R R R R R A
H-9	368-377	ProThrThrArgThrAspAspLysLeuArg B T R T T T T R R A
H-10	442-451	AspLeuTyrLysSerAsnHisAsnAsnVal R B B T R R T T T T

*The letter under each amino acid indicates contibution of one amino acid to the secondary structure of the entire molecule; T= turn, B= β sheet, A= α helix, R= random coil.

Production of a highly specific polyclonal rabbit antiserum against H has been earlier described by Lund and Salmi (1982). Reactivity of this serum with the peptides is shown in Figure 10. The same peptides were recognized by both the anti-H serum and the anti-MV serum. One of the peptides, H-9, was superior to the others in binding anti-H serum. Polyclonal anti-H bound to H-9 with high affinity to a titer of 2500, considered quite high as it binds to isolated H with a titer of approximately 20,000.

Experiments were done with MAbs against H protein. Seven MAbs were tested with all the peptides in direct EIA. None of the peptides were clearly recognized by any of the MAbs. In some cases, there was a trend to binding at low affinity but as reproducibility of these results was not good they are not presented here. Human sera including ten SSPE

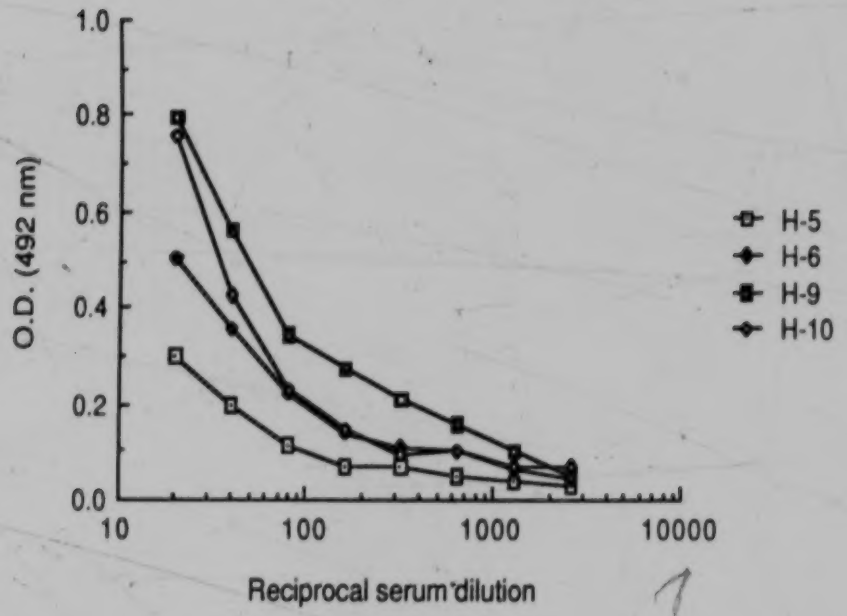

Figure 9. Binding of the polyclonal antiserum against purified MV to BSA-conjugated peptides. EIA microtiter plates were coated with 1 μg of each peptide-conjugate and the serum was serially diluted with two-fold steps.

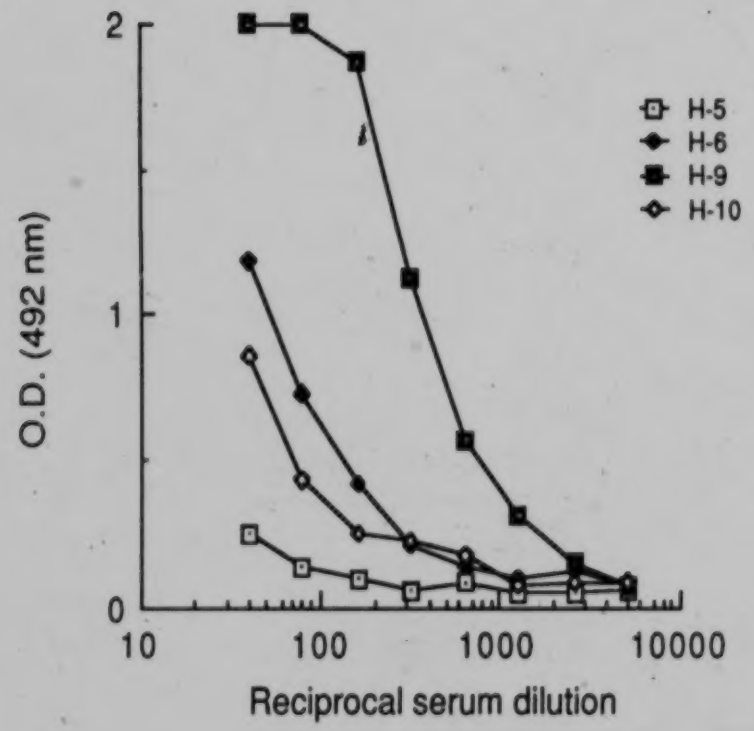

Figure 10. Binding of the polyclonal anti-H serum to BSA-conjugated peptides. Wells were coated with 1 μg of each peptide-conjugate and the serum was serially diluted with two-fold steps.

sera and ten pairs of acute-early convalescent sera were also tested. Again, certain sera bound to the peptides but the results were not consistent and are not presented here.

These results indicate that antibodies are elicited in hyperimmunization with intact virions or purified H against regions corresponding to the peptides synthesized in this study. However, the amount of these antibodies is at low level as only hyperimmune sera bound significantly to the peptides.

4.7 Production and characterization of antisera against the synthetic peptides

4.7.1 Kinetics of antibody elicitation

Antisera to the peptides were produced in rabbits by immunizing the animals with the KLH-conjugated peptides for seven months. Antibodies against the homologous peptide were present in serum three weeks after the first injection except in the rabbit immunized with the H-5 peptide. The titers against the H-6, -9 and -10 peptides increased for three months after which they reached maximum. However, the anti-H5 serum continued to increase up to five months after first immunization but it never reached comparable titers to the other peptides. As the antibody response can be unique depending on the genetic background, another rabbit was immunized for three months with the H-5 peptide. This animal had a similar poor antibody response as the first rabbit and was terminated.

Of interest is the quality of the antibody response after three months. Titers against purified virus and H present in cell lysate increased so that in the animals immunized with H9 and H6, the highest titers were seen in the five month samples. The antiserum to the H10 bound first time to the purified H in the seven month sample. Also, anti-H5 improved at seven months in binding to the purified virus. These results would indicate that very long immunization times are needed for some peptides to produce protein-recognizing antibodies.

4.8 Characterization of the reactivity of the antisera

The reactivity of the antisera with the H protein was tested in several assays. Firstly, the direct EIA with three types of antigens were used. Both preimmune and hyperimmune sera of each rabbit were included in the tests. When cell lysate antigen prepared from MV infected cells was used, only two of the sera, anti-H6 and anti-H9, recognized the H protein present in this preparation (Figure 11). Antisera to the H-9 bound with high affinity and good titer (20,000) to the H protein present in infected cells. The anti-H6 did also react moderately well with the H whereas anti-H5 and anti-H10 bound at the same level as preimmune sera. All the anti-peptide antisera did bind to purified virions (see Figure 12).

As the conjugation methods and linker sequence used to conjugate the peptides to the carrier, i.e. KLH, were the same, antibodies may also be produced against the linker region. To rule out the possibility that antibodies produced to the linker region or KLH do not cross-react with purified virus, a control antiserum produced against a similarly prepared peptide conjugate with a sequence corresponding to the nonstructural C protein of MV was used (Mäkelä and Salmi, unpublished data). This serum did not bind to the purified MV indicating that the binding of the anti-H sera to MV is a specific phenomenon. Unexpectedly again, the affinity of antiserum to H-10 was the best in binding to the purified virus in EIA and the titer was equal to anti-H9. Several different batches of purified wild-type MV were tested and in all cases the results were similar. The anti-H5 antibodies also bound to purified virus but with low titer. A further step was to test antisera on purified H isolated and purified using LL and affinity chromatography. As can be seen in Figure 13, antiserum to H-9 proved to strongly recognize purified H protein. In fact, its binding was equal to that of the polyclonal anti-H serum in this test. The anti-H6 and anti-H10 bound also to H but with an almost 10-fold lower titer.

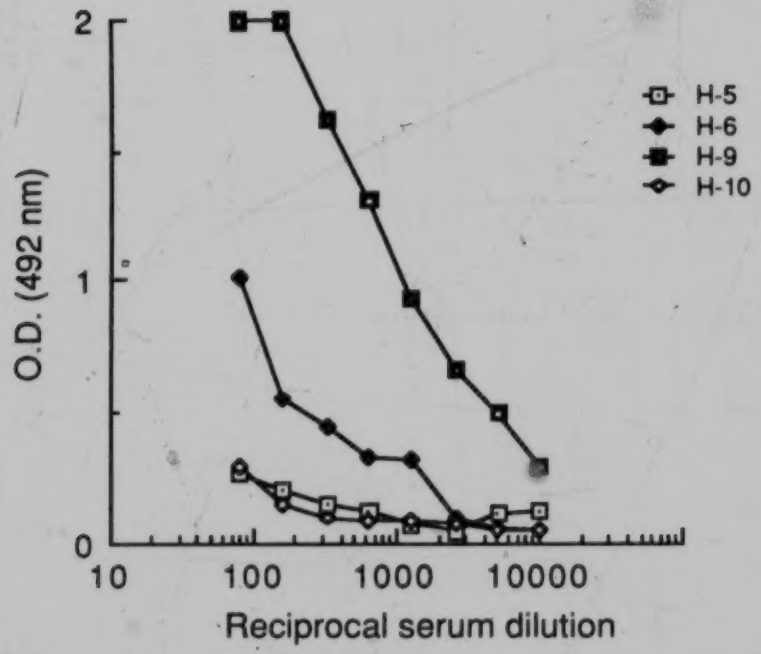

Figure 11. Reactivity of the anti-peptide antisera with lysate antigen prepared from MV infected cells in EIA. Wells were coated with 2 μg of the antigen and the sera were titrated with two-fold dilutions. Only H-6 and H-9 bound significantly to hemagglutinin present in infected cells.

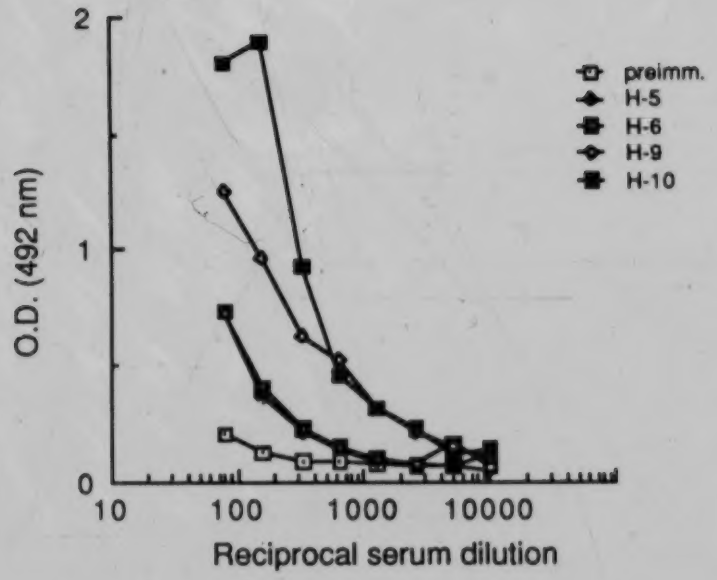

Figure 12. Reactivity of the anti-peptide antisera with purified MV antigen. Wells were coated with 0.3 μg of purified virions and sera were titrated as above.

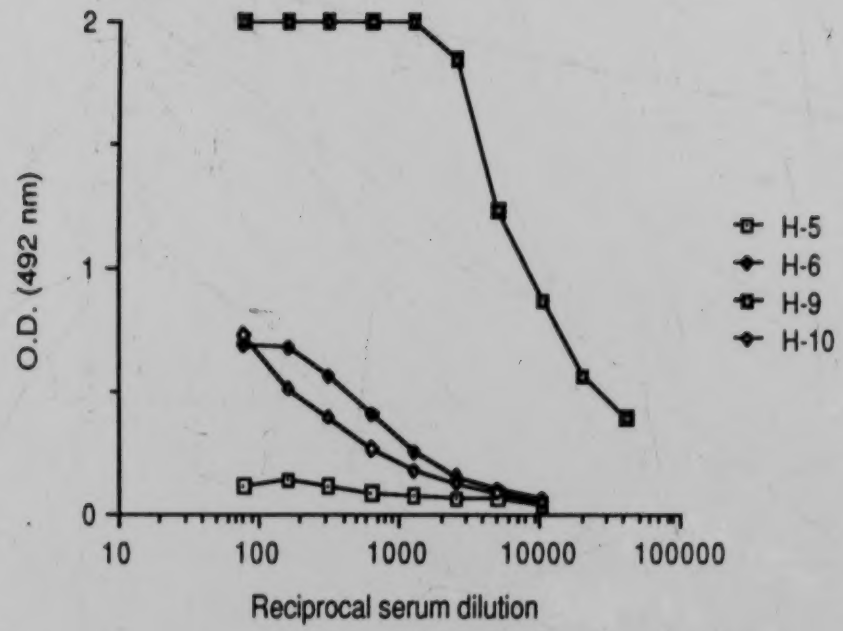

Figure 13. Reactivity of the anti-peptide antisera with purified MV hemagglutinin. Wells were coated with 0.1 µg of purified H and titrated as above. All antisera except H-5 recognized isolated H protein.

EIA results suggested that antisera to H-5 and H-10 would recognize mainly conformation dependent sites whereas H-6 and H-9 could recognize sites which are present in all conformational forms of the H protein. To further study this possibility, Western blot techniques were used. Purified MV was separated on SDS-PAGE and proteins transferred electrophoretically to nitrocellulose filters on which the immuno reaction was done. Supporting the EIA findings, anti-H6 and anti-H9 bound to denaturated H whereas anti-H5 and anti-H10 did not (Figure 14).

Based on these results, it was expected that anti-H9, and perhaps anti-H6, could precipitate H in radioactively labelled cell lysates using RIPA. However, only anti-H9 was able to do this as seen in Figure 15. This precipitation was done with nine week serum samples but seven month samples gave identical results (data not shown). There was a weak band in the anti-H10 lane which was clearly of lower molecular weight than H. This band was not seen in later RIPA and may represent non-specific precipitation. The fact that anti-H6 was not able to precipitate H is not surprising as the affinity of this serum in EIA was relatively low. The RIPA results confirmed the earlier experiments.

IF was also done as an additional test for antibody specificity. Both fixed and non-fixed MV infected Vero cells were used and controls included immune sera on uninfected cells and preimmune sera on infected cells. Only anti-H9 produced relatively bright fluorescence on fixed cells. This was seen mainly as small spots in the cytoplasm. In surface IF, anti-H9 reacted moderately and antiserum to H-10 somewhat more weakly (anti-H10 shown in Figure 16). The IF results are thus in accordance with those obtained in the other assays.

As all the peptides are located close to possible glycosylation sites, it was necessary to determine whether the binding of the antisera to H would improve if oligosaccharides were removed. To do this, virus was treated with endoglycosidase H. This enzyme efficiently cleaves high mannose glycans from glycoproteins.

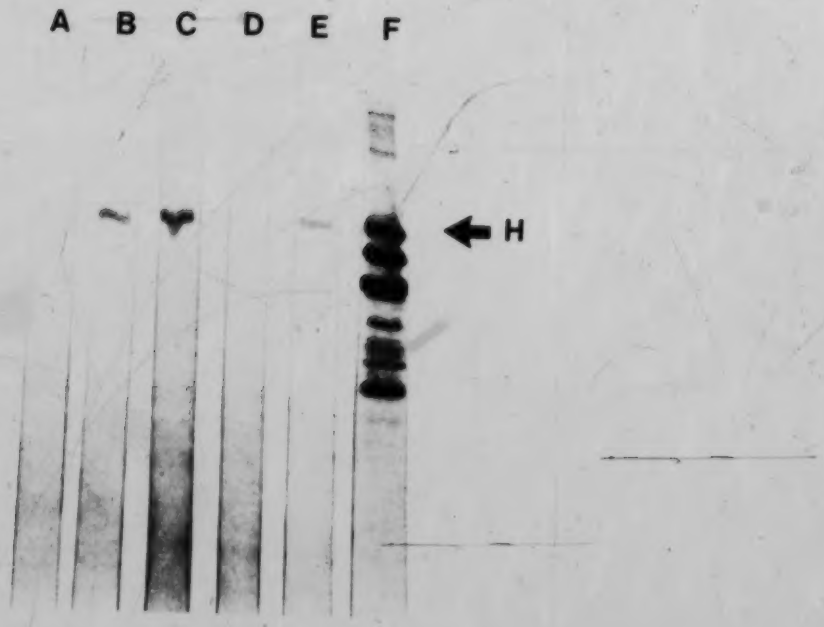

Figure 14. Western blot of anti-peptide antisera. Purified MV preparation separated in SDS-PAGE was transferred electrophoretically to nitrocellulose filter and the anti-peptide antisera were allowed to react with the filter. H refers to hemagglutinin.

(A) antiserum to H-5

(B) antiserum to H-6

(C) antiserum to H-9

(D) antiserum to H-10

(E) polyclonal antiserum to H

(F) polyclonal antiserum to whole virus

Figure 15. Immunoprecipitation of [^{35}S] -methionine labelled lysate antigen prepared from MV infected cells by anti-peptide antisera.

(A) polyclonal antiserum against H

(B) polyclonal antiserum against whole virus

(C) antiserum to H-5

(D) antiserum to H-6

(E) antiserum to H-9

(F) antiserum to H-10

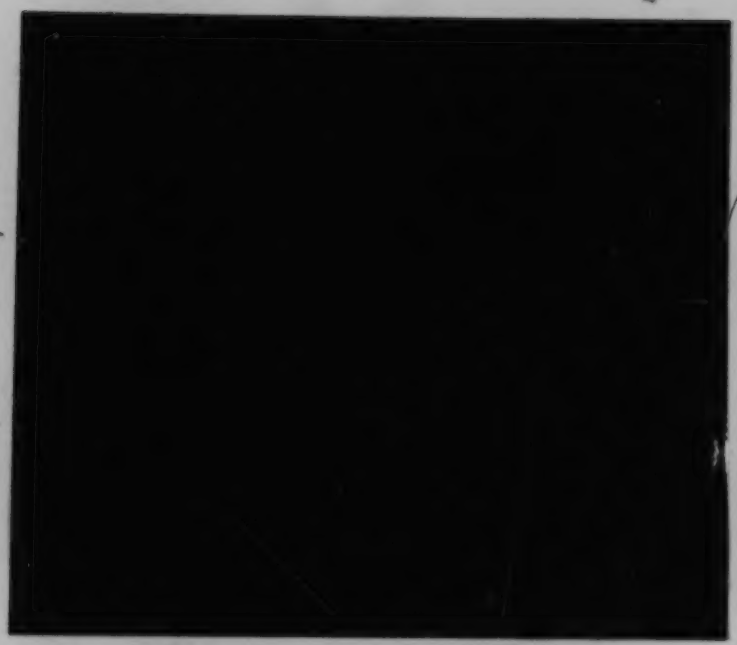

Figure 16. Immunofluorescence of non-fixed Vero cells with anti-H10 serum.

None of the antisera showed increased binding to virus after this treatment indicating that the sites against which the antibodies were formed are not masked by oligosaccharides. In fact, endoglycosidase H treatment slightly lowered the level of binding as compared to nontreated virus. Of interest is the observation that this untreated virus preparation bound normal rabbit sera non-specifically at low dilutions and this 'background' binding was considerably reduced after the enzyme treatment.

To test the biological activity of the sera, plaque neutralization and HI tests (Table 3) were used. None of the antisera had HI activity when tested against wild type strain. Titers obtained show a 50 % inhibition in number of plaques from preimmune to immune serum. The anti-H5 had the highest neutralizing activity although it did not bind very strongly to MV in EIA.

A summary of the reactivity of the antisera is given in Table 4.

Table 3. Titers of anti-peptide antisera in plaque neutralization and HI tests. NT titers showing 50% reduction in number of plaques is given.

	NT	HI
antiserum		
H5	80	16
H6	20	16
H9	40	16
H10	40	16

Table 4. Summary of the reactivity of the anti-peptide antisera in different immunoassays.

	EIA[a]			RIPA	WB	IF	
	1	2	3			fixed	non-fixed
Antiserum							
H5	+	-	-	-	-	-	-
H6	+	+	+	-	+	-	-
H9	+	+	+	+	+	+	+
H10	+	-	+	-	-	-	+

[a] EIA done on different antigens; 1= purif. virus, 2= cell lysate, 3= purif. H.

EIA= enzyme immunoassay, RIPA = radio immunoprecipitation, WB= western blot, IF = immunofluorescence

4.9 Mouse T cell proliferation assays to hemagglutinin

To study the role of the H protein in T cell proliferation assays, a mouse model developed by Ziola et al. (1987) was used. Balb/c mice were treated with cyclophoshamide two days before immunization with inactivated MV antigen (MV does not replicate in mouse cells, so in order to better define experimental conditions inactivated MV was used). Seven days post immunization, lymph nodes were removed, cells representing mainly the T cell population were isolated and challenged in vitro with different antigens. In the first set of experiments, H was enriched from plasma membrane preparations by LL chromatography (specific for high mannose glycoproteins including H). MV NCs were also purified and used for challenge. The purity of these preparations can be seen in the SDS-PAGE results shown in Figure 17. The H preparation seen in lane C was used in the first experiments.

A representative example of the results with the LL preparation of H is shown in Figure 18. Glycoprotein fractions from MV infected cells gave an almost equal response to whole virus whereas NC gave a very low response and VSV glycoprotein used as a control gave no response. In some experiments, NC gave a somewhat higher response and LL preparations elicited a higher response than the whole virus. The concentrations given on the Figure 18 represent the optimal concentration which induced the highest response of each antigen in this experiment.

As the LL preparation giving high response in the assays showed a major peak in H in SDS-PAGE, the H protein was further purified. As the first approach, H was cut from the gel and eluted as described in materials and methods. This preparation, however, was clearly less effective as a challenge antigen than the LL antigen. The results of one experiment are shown in Table 5.

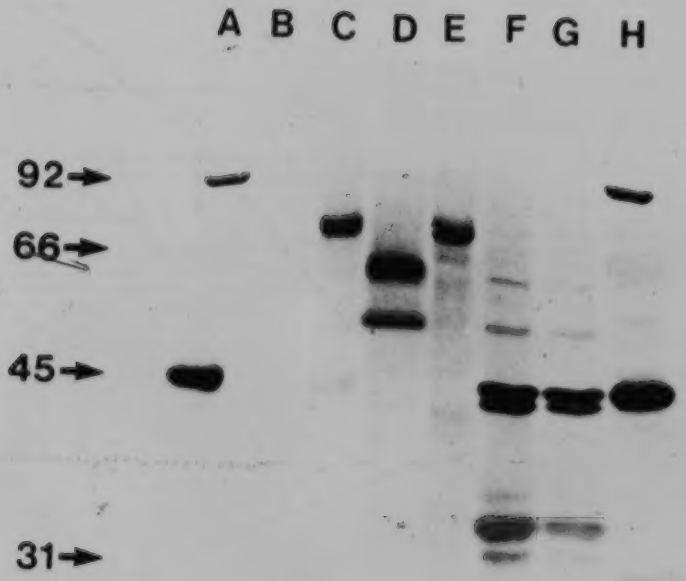

Figure 17. Purity of the viral antigens used in the T cell assays shown by SDS-PAGE with silver-staining.

(A) molecular weight markers

(B) none

(C) MV H after LL column purification

(D) VSV glycoprotein fraction

(E) MV H after both LL and affinity column purification

(F) MV nucleocapsid

(G) MV nucleocapsid

(H) molecular weight markers

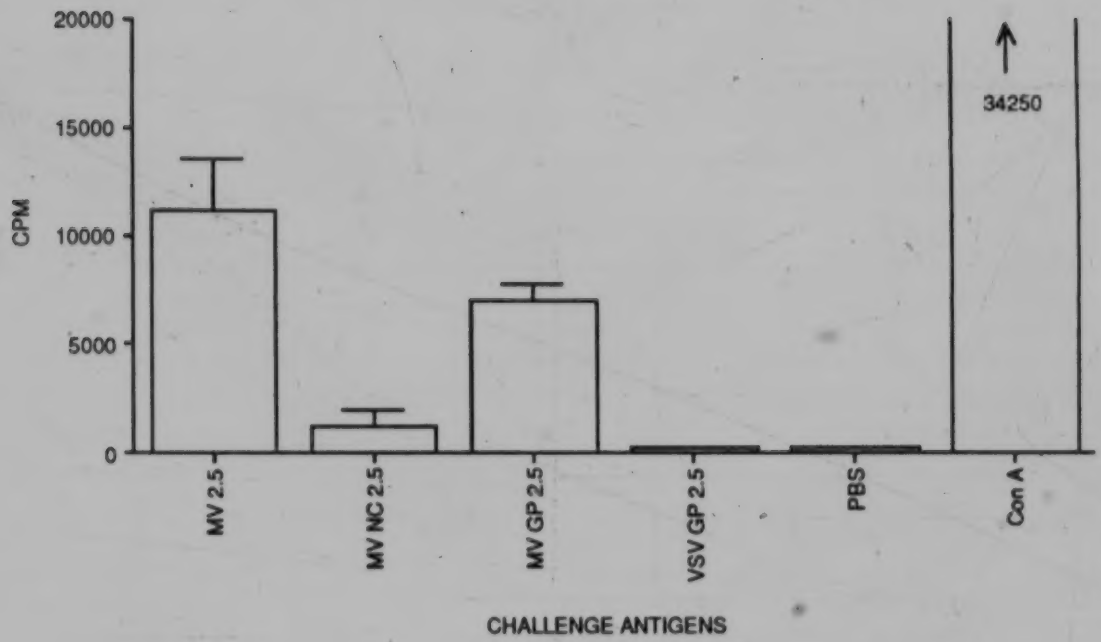

Figure 18. Challenge of MV immune lymphocytes with different antigens. Five mice were treated with cyclophosphamide (CP, 200 mg/kg) and immunized two days later with 8 μg of MV mixed with 100 μg of DDA. Seven days after, lymph nodes were removed, cells separated and challenged with different antigens in vitro. MV and VSV GP refer to the glycoprotein fraction isolated from virus-infected cells, NC is isolated nucleocapsid, Con A is concanavalin A and the numbers indicate concentration of the antigen (μg/ml).

Table 5. Challenge of MV immune lymphocytes with different antigen preparations. Five mice were pre-treated with CP and immunized with 8 μg of inactivated MV in DDA and seven days later lymph nodes were removed, cells separated and cultured for two days in the presence of stimulating antigens. Cultures done in quadruplicate were pulse-labeled for 16 h with 0.4 μCi [^{3}H]-thymidine/well .

Challenge Antigen (μg/ml)	Counts per minute ± S.D.
MV(5)	6317 ± 509
MV(2.5)	8025 ± 1220
VSV (5)	1067 ± 90
VSV(2.5)	1206 ± 477
H gel (2[a])	2201 ± 845
H gel (1)	1115 ± 156
gel contr. [b]	330 ± 50
NC(5)	3004 ± 301
PBS	187 ± 77
ConA	52765 ± 325

[a] H gel refers to the protein eluted from SDS-PAGE; concentration of this preparation was barely detectable with Bio-Rad microassay so this is an estimate

[b] piece of acrylamide 'eluted' the same way as the H preparation

The results suggested that H in an purified monomeric form would not elicit as good a response as that isolated from membranes. However, there was a chance that the concentration of the gel derived material was not high enough. The yield eluted from the gel was very low so it was not practical to concentrate the preparation. As LL chromatography was shown to produce high enrichment of H, affinity chromatography employing Sepharose coupled MAbs against H was used combined to LL chromatography. This two-step method was highly efficient in purification of H protein as seen in Figures 17 (silver staining of SDS-PAGE) and 19 (Western blot before and after affinity column).

Hemagglutinin as other glycoproteins has a tendency to form aggregates when purified and can be less immunogenic as a result.. To avoid this problem, purified H was inserted into liposomes in order to mimic the natural state of the H on the viral membrane. The assumption proved correct as shown in Figure 20. H liposomes clearly elicited a higher response than an optimal dose of whole virus. To show that this was not a lipid/adjuvant effect, VSV glycoproteins were inserted into liposomes as a control. Liposomes were made with NC preparations to study the effect wih an internal MV antigen. The response was somewhat less than to the normal NC preparation and can be explained by the hydrophobic nature of the nucleoprotein (NP), i.e., NP would not be exposed on the outer surface of liposomes but is rather inside.

As mentioned above, the protein eluted from the gel did not induce a high response, possibly explained by strong aggregation. To study this further, part of the purified H was treated in the same way as the liposome preparation with identical protein amounts except that no lipids were added. Liposomes were then compared to the H aggregates as challenging antigens. Figure 21 shows the results of the experiment. Liposomes were superior to the aggregate form of the H in stimulation of MV immune lymphocytes.

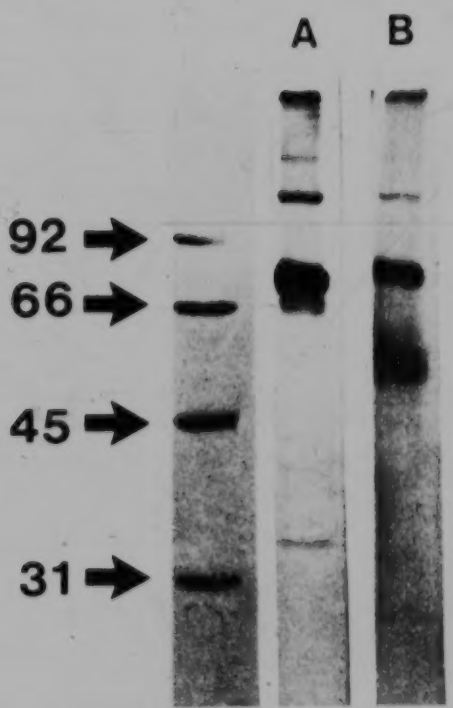

Figure 19. Western blot of the H preparations. (A) shows the preparation after LL chromatography and (B) LL followed by affinity chromatography.

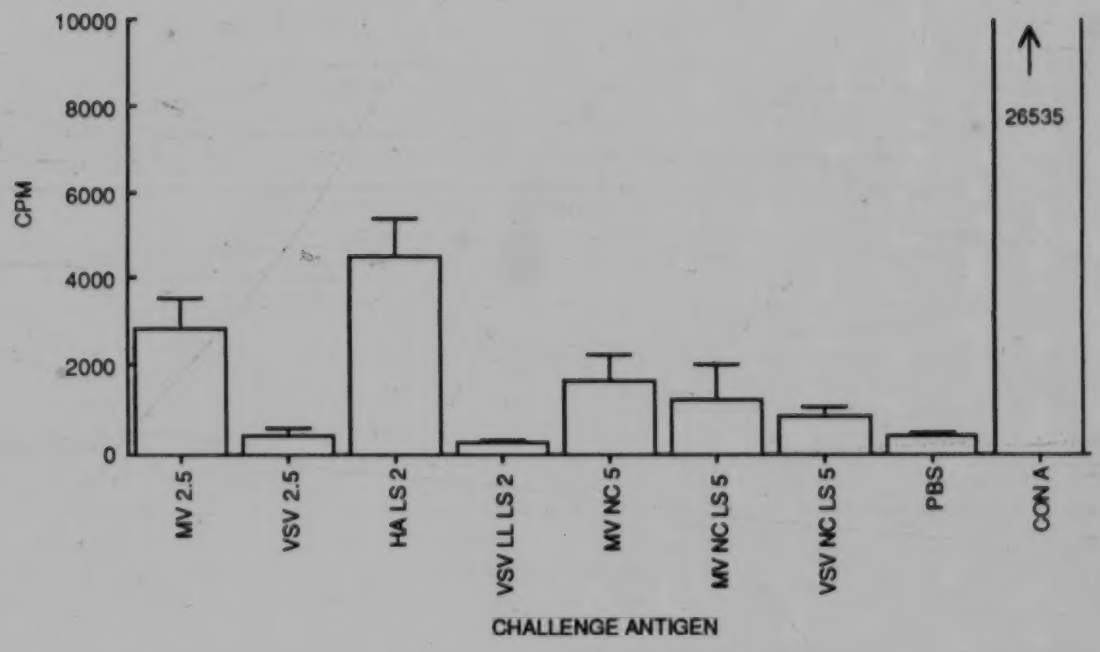

Figure 20. Effect of incorporation of antigens into liposomes in secondary challenge of MV primed lymphocytes. LS refers to liposomes, LL to lentil lectin isolation of the glycoprotein fraction from VSV and MV infected cells, other symbols as above.

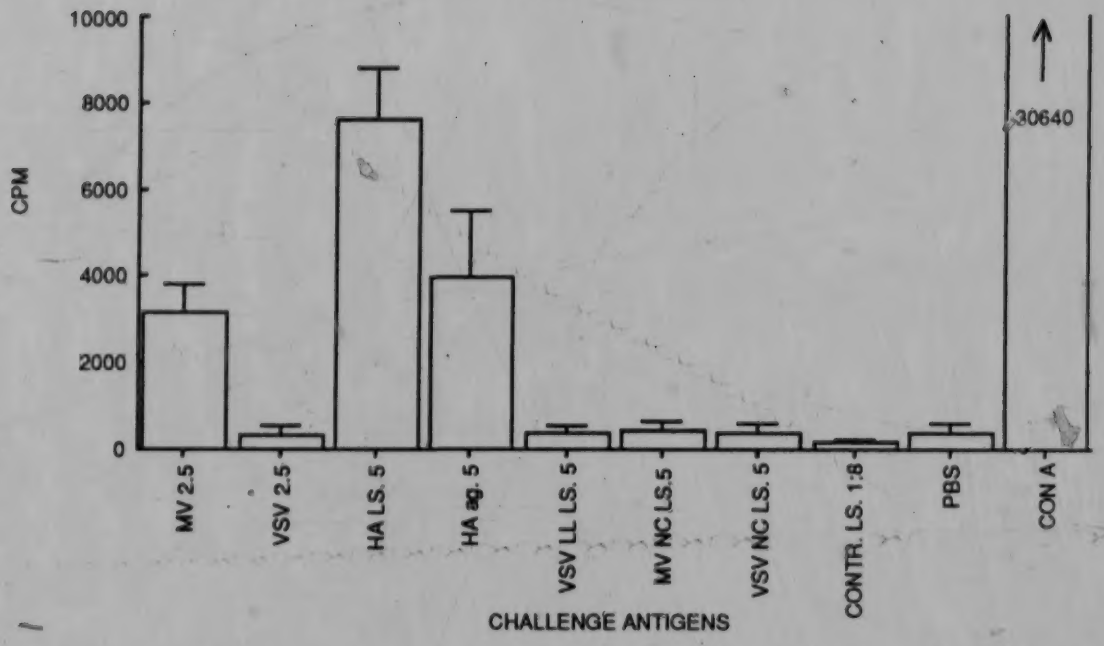

Figure 21. Immunogenicity of purified hemagglutinin incorporated into liposomes versus H aggregates. HA LS refers to liposomes, HA ag to aggregates of pure HA and control LS to lipid control.

4.10 T cell assays with the synthetic peptides

To study the T cell response to the H protein at a molecular level the synthetic peptides previously described were used. Several experiments were performed with the conjugated peptides but this proved to be a system with great variations and the results were inconsistent. An inherent problem is that the amount of peptide (w/w) even in the best conjugates is only one fifth of the carrier. Thus, when high concentrations are required, as often is the case with synthetic peptides in T cell assays, such levels are impossible to reach. Therefore, two of the peptides (H-6 and H-9) which reacted best in the antibody assays were obtained in a non-conjugated form as well. In preliminary experiments, mice were immunized with increasing amounts of the free peptides either in FCA or in DDA. The number of cells cultured in vitro was varied and different culture times were tried to find the optimal conditions for these assays. These experiments indicated that firstly, there was no difference between these two adjuvants in inducing the response. DDA was employed in most experiments as the immunizing solution is easier to prepare and DDA does not cause necrosis of the skin in the animals when injected intracutaneously . Secondly, the required amount of the peptide for immunization was at least 30 μg/mouse. Thirdly, the number of cells per well had to be relatively high, at least 300,000/well. Finally, pulse-labeling was optimal either on day three with overnight lower activity (0.5 μCi) labelling or on day four with a six-hour pulse-label using 1.0 μCi/well.

As no conclusive results were obtained from the experiments with conjugated peptides only free H-6 and H-9 were used in the final experiments. Neither could stimulate MV primed lymphocytes to proliferate even at concentrations up to 200 μg/ml. However, when either H-9 or H-6 were used as priming antigens, lymphocytes isolated from these mice proliferated in response to purified virus. Results of two experiments with H-9 are seen in Table 6. Again, no stimulation was seen with homologous peptide.

Table 6. Stimulation of H-9 primed lymphocytes with different antigens. In experiment 1, mice were immunized with 100 μg of free peptide in FCA and lymph nodes removed seven days later. Cells were cultured for three days followed by overnight pulse-labelling with 0.5 μCi [^{3}H] thymidine/well. In experiment 2, conditions were the same except that mice were immunized with 50 μg of free peptide in DDA.

		Counts per minute ± S.D.	
		Experiment 1	Experiment 2
Antigen	(μg/ml)		
MV	10	2208 ± 588	2362 ± 687
MV	2.5	1444 ± 148	1747 ± 741
VSV	10	532 ± 71	535 ± 293
VSV	2.5	ND*	591 ±393
Free H-9	50	508 ±102	320 ± 163
PBS	--	312 ± 98	750 ± 113
ConA	5	95328 ± 2005	16477 ± 1316

* not done

To show that this phenomenon is specific stimulation of lymphocytes by challenge antigens, several experiments were done with mice immunized with PBS and DDA. The experimental conditions were kept the same and the cells were challenged with the same antigens as cells primed with the peptides. No proliferation was observed in control cells except with concanavalin A(ConA; data not shown).

Lymphocytes present in lymph nodes consist mostly of T cells so these are normally used in proliferation assays. However, in order to make sure that proliferation seen in these experiments is not due to B cells, spleens were taken from peptide immunized mice and lymphocytes isolated on a ficoll gradient. These cells were deleted of

B cells using an anti-mouse immunoglobulin-coated beads column through which the cell suspension was passed. Table 7 shows results of an experiment with H6 immunized mice of which both lymph nodes and spleen were taken and B cells deleted from the spleen cell suspension. The same amount of cells per well was used with both preparations. In both populations, the priming phenomenon was clear. However, a few differences were observed. Firstly, in spleen cells purified H could elicit a response although lower than with whole virus. Secondly, background counts were higher in spleen cells and more variation was seen. Both findings could reflect the fact that there are differences in cell populations present in spleen and in lymph nodes.

Immunization with the H-9 peptide gave similar results (data not shown). These results suggested that peptides can prime for secondary challenge with whole virus. To study the ability of the peptide to induce another type of response, DTH assay was used. This method has been described by Smith and Ziola (1986) for MV. In the MV DTH assay, mice are pretreated with CP and immunized two days later with inactivated MV. After seven days, mice are challenged intracutaneously in the footpads with antigen and footpad swelling is measured 24 h later. With the peptides, CP treatment was omitted and mice were immunized with 25 μg of free peptide in DDA. A week later, they were challenged with 20 μg of either MV, VSV or the peptide. Results of one experiment are shown in Figure 22. This experiment was repeated once and similar results were obtained indicating that in the DTH assay H-9 primed mice respond to a secondary challenge with whole virus. At the time of preparation of this thesis only one experiment has been done with H-6. In this experiment, when mice were challenged with MV, footpad swelling was greater than that induced by H-9.

Table 7. Comparison of H-6 primed spleen and lymph node cells in secondary stimulation. Three mice were immunized with 100 µg free H-6 in DDA i.c. and seven days later lymph nodes and spleens were removed. B cells were deleted from spleen cells and both cell preparations were challenged with same antigens.

		Counts per minute ± S.D.	
		lymph nodes	spleen
Antigen	(µg/ml)		
MV	5	1973 ±1248	1705 ± 136
MV	1	166 ± 76	604 ± 440
VSV	5	141 ± 146	581 ± 509
VSV	1	161 ± 40	354 ± 127
H LS[a]	1	152 ± 35	741 ± 925
VSV LL LS[b]	1	141 ± 64	98 ± 11
H6	200	86 ± 28	176 ± 119
PBS	--	133 ± 30	117 ± 29
ConA	5	3348 ± 1258	22576 ± 9699

[a] H LS refers to liposome preparation of purified H, [b] VSV LL LS to liposomes of LL isolated glycoproteins of VSV infected cells.

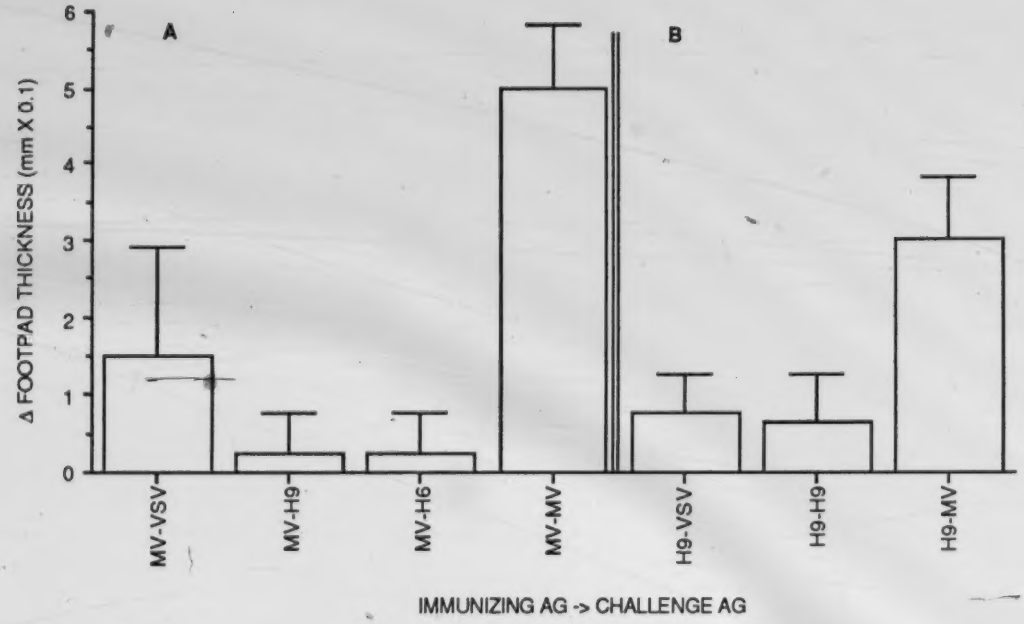

Figure 22. Delayed type hypersensitivity reaction induced in mice with MV (A) and free H-9 peptide (B). The mice in measles group were pretreated with CP and two days later immunized with 8 μg of MV mixed with DDA. Seven days later mice were challenged with 8 μg of virus in footpads. The other group of mice were not CP treated and were given 25 μg of free peptide followed by challenge in the footpad with 20 μg of peptide or MV after 7 days. Footpad swelling was measured after 24 h.

5. DISCUSSION

5.1 Competitive assays

In the first part of the study, a competition binding EIA to measure polypeptide- and antigenic site-specific antibodies against fusion and H proteins of MV was developed. The assay proved to be useful in detection of these antibodies in serum specimens. The assay was able to detect specific antibodies against at least two different antigenic sites on the fusion protein and one on the hemagglutinin protein.

The specificity of the assay for measles H and F proteins was demonstrated by allowing previously characterized MAbs against these polypeptides to compete with each other. Practically no cross reactions were found between the antibodies to H and F proteins.

The developed assay offers obvious advantages over the previously used methods for measuring antibodies to single viral proteins and antigenic sites. First, the method does not require purification of the polypeptides which is time-consuming and often leads to at least partial denaturation and disappearance of some epitopes. In methods such as western blotting the antigens are denaturated which definitely changes the antigenicity of the polypeptides. In the developed method either infected cell lysate or purified virions can be used as antigen on the solid phase both of which should be closer to the native form the proteins. Second, it is possible to quantitate the specific antibodies and express the amount in titer values. By using standard curves with known amounts of antibody it is possible to quantitate the immunoglobulins in sera as micrograms per milliliter. Recently, similar assays have been described for murine hepatitis virus-4 (Talbot et al., 1985), herpes simplex (Ross et al., 1985), influenza A (Wang et al., 1986), *Mycobacterium tuberculosis* (Ivanyi and Sharp, 1986), and hepatitis B (Waters et al., 1986; Waters et al., 1987). Talbot et al. (1985) directly labeled MAbs with peroxidase enzyme and used these in EIA system. However, peroxidase labeling in some cases dramatically reduces the antibody

activity, likely because of the large size of the molecule. Ross et al. (1985) did competitive assays with antibodies against herpes glycoproteins C and D using human sera. However, in this study, specificity of the assay for measuring antibodies to two different proteins was not demonstrated. Cross-inhibition studies between mouse MAbs cannot be done unless another detection method other than anti-mouse immunoglobulin is used for the indicator MAbs. In studies by Ivanyi and Sharp (1986) and Waters et al. (1986) either radiolabelling or peroxidase labeling of the MAbs was used. The method developed in the present study has two advantages compared to those used previously. Firstly, labeling of MAbs with biotin ester, a small molecule, preserves antibody activity well. Therefore, affinity constants of the antibodies are not changed making the method more reliable and sensitive. The second advantage is that radioactive material is not needed.

To compare the relative amount of different antibodies in different serum groups antibody titer ratios were calculated. In anti-F/anti-H antibody titer ratios, convalescent sera had significantly higher values than normal blood donors or SSPE patients showing that the levels of antibodies to antigenic sites on different polypeptides do not increase at corresponding rates after infection. There was a difference between NBDs and SSPE patients in one of the ratios defined by two MAbs to F protein. This ratio varied slightly within groups. In immunized mice a difference between strains in the same ratio of titers against F protein was noticed. Another finding was that though Balb/c mice had significantly higher titers to all sites on the fusion protein, the titers against the antigenic site on the H in SJL mice were at about the same levels. These results suggest that the antibody response to different proteins and even to single antigenic sites on the same polypeptides are independent phenomen.

In measles as in many other viral diseases, antibodies against certain polypeptides may have an essential role in the outcome of the disease (Rammohan et al., 1982). As well, only some epitopes on viral proteins may be important in protective immunity and in determining the pathogenicity of the virus, as has been shown with some animal models

(Dietzschold et al., 1983; Talbot et al., 1985; Löve et al., 1985). Therefore, tests and other applications of the method described here are needed for studies of viral pathogenicity when site-specific responses to viruses are analyzed.

5.2 Synthetic peptides

In an attempt to analyze the antigenicity of MV H, four peptides were synthesized in this study. Thus far, no information is available about the three-dimensional structure of H or localization of antigenic sites on the molecule. As a result, a prediction program for selection of regions to be synthesized was used. It must be emphasized that in this kind of analysis, no parameters used can accurately predict antigenic sites on proteins. Therefore, surface exposed areas can be revealed with a certain statistical probability of less than 100%. Specificity of these prediction programs is not particularly good as the results usually give more sites than the molecule can actually have and some 'real' sites are not predicted at all. These facts present the background problem when the peptide approach is used for antigenicity studies.

All sites corresponding to the peptides that were used in this study are likely to be exposed on the surface of the H. This was supported by several findings. Firstly, all peptides were recognized by polyclonal antisera to whole virus although not strongly. The antiserum bound most weakly to the H-5 peptide. This peptide has a cysteine in the middle which might explain its poor immunogenicity as the cysteine probably forms bridges between the peptide and the carrier molecule. Three of the peptides, H-6, 9 and 10, also bound antibodies raised against purified H further confirming that these sites must be immunogenic at least when hyperimmune sera are made.

Characterization of the antisera produced to the peptides resulted in several observations. All peptides were immunogenic, ie., capable of eliciting homotypic antibody. However, it was apparent that antibodies elicited against the H-9 peptide

(residues 368-377) were superior in recognizing H in all tests. The tests suggest that this determinant would be exposed on monomeric H. This claim is based on several facts. Firstly, this peptide significantly bound antibodies present in the serum against purified H. The titer of this serum on conjugated peptide was approximately 2500 versus 20000 on the purified H so the antibodies recognizing the peptide represent a large part of the total antibodies. Secondly, antiserum to H-9 bound to lysate antigen prepared from infected cells (in which a large part of H is still in the cytoplasm as a monomer) with an almost 10-fold higher titer than to purified virus (on which H is probably a dimer or trimer). Further, there was a five fold difference in titers between the serum versus lysate or purified H protein. In addition, this antiserum recognized H both in western blots and radio immunoprecipitation. In Western blots the protein is in denatured form whereas radio immunoprecipitation most likely measures similar binding to that of lysate antigen in EIA. When purified virus was disrupted with SDS and coated on EIA plates, the binding of anti-H9 serum strongly improved (data not shown). These findings indicate that this site is partly masked in purified virus where it could form a part of the interface area between the monomers. None of the MAbs showed clear binding to this peptide but this could be explained by the fact that MAbs used in this study have been raised against virions. As polyclonal anti-virion serum did not recognize this peptide nearly as well as anti-H serum, the interface site possibility could be right.

The H-6 peptide (residues 235-245) had similarities to H-9 as it bound antiserum to H better than anti-whole virus antibodies. Antibodies elicited against H-6 recognized hemagglutinin in sensitive tests (EIA, western blot) but did not precipitate the protein or show fluorescence. There was no significant differences in binding to H in lysate, purified virus or as a pure protein. Therefore, it is probable that this site is not important in immunogenicity of the protein although it most likely is surface exposed.

The peptide H-10 (residues 442-451) was different from the others in several ways. Polyclonal antiserum to whole virus bound equally well to H-10 as to H-9 as did anti-H

serum. However, antiserum elicited against H-10 gave unexpected results. It bound relatively early during immunization to purified virus with a high affinity. This was tested with several different batches of purified wild type virus and results were always similar. However, none of the samples taken during seven months of immunization showed binding to cell lysate antigen. Of interest is that the serum bound to purified H only in the last seven-month sample. This suggests that a long immunization is needed with some peptides to produce protein-reactive antibodies. Antibodies bind to H present as a native form but not in denatured (western blot) or immature form (cell lysate). This indicates that the antibodies are formed against conformation-dependent sites. It is, however, paradoxical that a short peptide can elicit antibodies against sites that may be folded in a quite different way on the intact protein. Wright et al. (1986) call this concept disorder-order phenomenon, i.e., antibodies against disordered peptides recognize ordered proteins. There are two explanations for this: either proteins are more flexible than is assumed or short peptides are more structured. Wright et al. (1986) have presented evidence from nuclear magnetic resonance observations that a nine amino acid peptide in water solution could have secondary structure. On the other hand, it is a general assumption that peptides could have several conformations in solution (Lerner, 1982). Provided this is true, the assumption is that one of these conformations approximates the one adopted in the intact molecule. However, as only some conformations represent the state of lowest free energy, peptides would adopt the conformation simulating the one in the native protein only for a short time. This would also explain the low antibody titers often seen with anti-peptide antibodies.

The H-5 peptide proved to be the least interesting in this study. Although it was recognized by antiserum to whole virus, this was questionable and by no means indicative of strong immunogenicity of this site in the native molecule. The antiserum raised against H-5 did confirm that the site likely is exposed on the surface of the protein as it bound to purified virus. However, in all other tests this serum showed no reactivity. Of interest

would be a substitution of the cysteine in position 188 since another sequence of Edmonston strain H which was available for our use (W. Bellini et al., unpublished) had tyrosine. However, the Hallé strain has a cysteine in this position according to the study by Gerald et al. (1986).

Three of the peptides studied (H-5, -6, -10) contain asparagine residues which are potential glycosylation sites (N-linked), with H-10 having two asparagines. The effect of carbohydrate removal was studied using endoglycosidase H, an enzyme which cleaves high mannose sugar complexes from the protein backbone. This treatment did not significantly change binding of any of the antisera. These results are in contrast with Alexander and Elder (1984) who found dramatic changes in binding of most antibodies studied. As glycosylation of MV H is not properly characterized, it is difficult to say how much of the sugar moieties were actually cleaved.

As has become apparent from the results section, the peptides that were synthesized in this study were immunogenic even in terms of raising virus-recognizing antibodies. However, a question should be asked about the possible biological significance of these peptides, i.e., whether any represent the immunodominant antigenic site. Based on results derived from binding of human sera the answer would be no as none of the sera significantly bound to the peptides. However, the answer may not be so simple. First of all, the peptides used in the present study were 10-11 residues long. Although generally speaking this should be a long enough determinant for the B cell receptor, i.e. immunoglobulin, there is always the chance that the next residue from either end could have been the one dramatically enhancing the immunogenic activity. It has been shown that only one amino acid can be crucial in determining the total immunogenic activity of a peptide although it is not enough to form a determinant by itself (Kilgannon et al., 1986). At least in the case of the H-9 peptide, the site seemed to be highly immunogenic even when native protein was used as immunizing antigen. Therefore, it was somewhat surprising that human sera did not bind to the peptide. On the other hand, it is generally

accepted that antibodies mainly recognize discontinuous determinants (Benjamin et al., 1984), thus, the failure of human sera to bind to the peptides is not surprising. Geysen et al. (1986) claimed to delineate a peptide simulating a conformational discontinuous determinant but whether this can be generally applied is not known. Another factor which may have effect in these studies is the orientation of the peptide to carrier. Dyrberg and Oldstone (M. Oldstone, personal communication) have shown that the immune response to identical peptides can be different depending whether the peptide is coupled to the carrier either at the amino- or carboxy-terminus.

5.3 T cell assays

The purpose of the T cell assays was to examine the role of H in proliferative assays and to delineate the antigenicity of the H protein. Man is the natural host for measles but the problem is that lymphocytes of most seropositive humans do not respond to MV in proliferative assays. Consequently, a mouse model developed by Ziola et al. (1987) was used. Since MV does not replicate properly in mice, inactivated virus was used for immunizing the animals to clearly define experimental conditions. In this model, mice are treated with CP two days before immunization with antigen mixed in DDA adjuvant. The combination of these two compounds, CP and DDA, has been shown to potentiate the cellular response either measured by DTH or blast transformation assays (Smith and Ziola, 1984; Smith and Ziola, 1986; Ziola et al., 1987).

As a control in all experiments, similarly prepared antigens of vesicular stomatitis virus were used. This virus was selected as a control for several reasons. Firstly, it buds from host cells in the same manner as MV. Secondly, as it can be grown in Vero cells, a similar composition of host cell derived components is expected. Finally, no cross-reactivity between MV and VSV has been reported. Purified VSV gave in most assays a response which was above background. This is assumed to be derived from lipids and

contamination by cellular proteins. However, in all cases the VSV response was essentially lower than elicited by MV showing the specificity of the assay. When components were further purified, the challenge response was in most cases at background levels.

The first set of experiments with MV immunized mouse lymphocytes suggested that the glycoprotein fraction of MV infected cells isolated by LL chromatography includes major antigens in a proliferative assay. When this preparation was separated by SDS-PAGE, a major polypeptide was seen at the area where H was expected. In Western blots, H protein was the major antigen present in the preparation. This led to experiments using further purified H. First, H protein was eluted from polyacrylamide gel and used as a challenging antigen. The response, however, was clearly lower than with the LL preparation. The low yield of the H component obtained by elution from gel necessitated the use of affinity chromatography combined with LL chromatography to produce highly purified H as shown by SDS-PAGE and Western blot. Purified glycoproteins have a high tendency to aggregate which results in reduced immunogenicity. Consequently, purified H was inserted into liposomes and an equal part was treated the same way without the addition of exogenous lipids. These antigens were then compared as challenging antigens. The results showed that liposome H is superior to aggregated form in eliciting a response in vitro.

Bellini et al. (1980) purified H with affinity chromatography and tested lymphocytes of three high-responders to MV with this H preparation. In two out of three cases, plasma membranes isolated from MV infected cells gave a higher response than purified H and in one case the response was almost equal. These findings suggest that either F protein is important in eliciting the response or alternatively, the form which purified H adopted was not as immunogenic as the native form in plasma membranes. Rose et al.(1984) further studied this by eluting antigens from a polyacrylamide gel. The same high-responding patients were used in the study which suggested that both H and F proteins were the major stimulating antigens. However, the response obtained with the gel

eluted H was clearly lower than that to affinity chromatography purified in their previous study. The findings in the present study are in accordance with the reports by Bellini et al. (1980) and Rose et al. (1984). In the mouse system it was also found that LL-affinity chromatography purified H was more immunogenic than that eluted from polyacrylamide gel. After affinity chromatography, a major part of the antigen is probably still in dimeric form whereas a monomer is eluted from the gel. This could have an influence on the immunogenicity of the antigen. It is generally accepted that T helper cells recognize short fragments of antigen in the context of MHC molecules rather than the native form. However, prior to recognition, the antigen has to be processed and presented. Since these steps of the immune recognition cascade are still largely unknown, the form of the antigen when encountered by antigen presenting cells (APC) could have an influence on the final processing. In fact, insertion of antigen into liposomes has at least an in vivo effect on the immune response (Thibodeau et al., 1984). The present study would suggest that this may also be true in vitro. In the liposome, H probably adopts a more native form and it could be processed and presented to APCs more effectively to elicit a higher response.

Synthetic peptides were used in this study to analyze the antigenicity of the H subunit in T cell recognition at a molecular level. Preliminary experiments were done with conjugated peptides by immunizing the mice with KLH-conjugated peptide and challenging then with BSA-conjugate or vice versa. This approach proved to be, however, not ideal. First of all, the conjugation ratio of peptide to carrier is usually between 5-15. If the molecular weight of a peptide is for example 1.5 Kd and that of carrier 60 Kd and conjugation ratio eight, this would mean that the weight ratio in a peptide conjugate would be 1:5 (peptide:carrier). To reach concentrations high enough for peptides to elicit a response (up to 200 µg/ml for non-conjugated) is practically impossible. As a result, free peptides were used in further experiments. As H9 and H6 gave most encouraging results in antibody assays these peptides were prepared as non-conjugated ones. Both of these peptides were first tested for their ability to elicit a secondary response of MV primed

lymphocytes. Neither of the peptides gave a response in these assays. Next, peptides were used to immunize mice and lymphocytes isolated from these were then challenged with different antigens. Again, it was apparent that neither one of the peptides could elicit a response even when lymphocytes primed with the homologous peptide were used. Of interest, however, was that a response to purified measles virus was seen. In control experiments, lymphocytes from PBS and DDA immunized mice were challenged with the same antigens and no proliferation was seen. To study this priming effect further, a DTH assay was used. In this assay it was also shown that at least H-9 and probably H-6 as well could prime mice for secondary challenge with MV.

The phenomenon that a short peptide can prime for proliferative response with a longer peptide (in this case, intact protein) has been described before. Singh et al. (1980) used TNP-peptides (trinitrophenyl haptenic group linked to a repetitive peptide) to determine the minimum length of a peptide necessary for priming and eliciting antibody, proliferative or DTH responses. They found that a nine amino acid peptide could prime the mice for antibody and proliferative responses whereas 12 amino acids were required for priming of DTH. In all cases, eliciting of the response required a longer peptide. The shortest peptide which could both prime and elicit the response was 18 residues. Although there are differences between peptides and these rules can not be generally applied, results obtained in the present study are in agreement with those of Singh et al. (1980).

The priming doses and optimal challenge concentrations used in this study correspond to those reported in the literature (Hackett et al., 1985, Milich et al., 1986). It seems to be a general phenomenon that there is a significant difference in molar concentrations required to elicit a response. Hackett et al. (1985) immunized mice with a decapeptide derived from influenza A hemagglutinin and made T cell clones. The stimulatory efficiency of different antigens to induce proliferation of the clones was tested. It was found that, on a molar basis, there was a million-fold difference between purified virus and the peptide in concentration needed to stimulate the clones . There was also a ten-

fold difference between purified H and whole virus in eliciting the response, i.e. the concentration of the purified virus needed to stimulate equal response to purified hemagglutinin was one tenth of that of the H protein. In the present study the concentration of purified H used was probably too low to elicit an equal response to whole virus although this was significantly higher than the control reesponse with VSV glycoproteins.

It should be noted that though a priming effect was seen in this study, the responses were generally rather low. However, the effect was seen in two types of assays suggesting that it is a real effect.

6. CONCLUSIONS

In the first part of the study a specific and sensitive method for analyzing polypeptide- and antigenic site-specific antibodies was developed. This method proved to be valid in studying the antibody response to measles virus at a detailed level. The results obtained with sera from different disease conditions suggest that there are differences between acute and chronic infection in the humoral response.

In the second part, antigenicity of measles hemagglutinin was studied in humoral immunity by using synthetic peptides representing possible antigenic domains. One of the four peptides seemed to be an important determinant in the isolated hemagglutinin and it was suggested that this could be in a di- or trimer complex part of the interface area. Otherwise the results showed that the qualitative antibody response to different peptides can be quite different although similar methods are used for prediction of the sites, synthesis and conjugation of the peptides.

The third part of the study examined the antigenicity and the role of hemagglutinin in T cell recognition. The results showed that although T cells do not recognize free antigen, the form in which antigen is seen by the cell-mediated system is crucial. The synthetic peptides that were used could prime for secondary challenge with whole measles virus both in proliferation and DTH assays. This would indicate that the H-9 region peptide could have biological importance in immune defense.

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